# CHARACTERIZATION OF THE ROLE OF ACID CERAMIDASE IN ADRENOCORTICAL STEROID HORMONE BIOSYNTHESIS

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Characterization of the role of acid ceramidase in adrenocortical steroid hormone biosynthesis

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**1dSa** – 1-deoxysphinganine
**ACTH** – adrenocorticotropin hormone
**AP** – activating protein
**ASAH1** – acid ceramidase
**ASAH1$^{KD}$** – ASAH1 knockdown H295R cell line
**ASAH2** – neutral ceramidase
**ACER1-3** – alkaline ceramidase, isoforms 1-3
**Bak** – Bcl-2 homologous antagonist killer
**Bax** – Bcl-2-associated X protein
**Bcl-2** – B-cell lymphoma 2 protein
**Bt$_2$cAMP** – dibutyryl cAMP
**C1P** – ceramide-1-phosphate
**CamK** – calcium/calmodulin-dependent protein kinase
**DBD** – DNA-binding domain
**CBP/p300** – CREB-binding protein
**CDK** – cyclin-dependent kinase
**C/EBPβ** – CCAAT-enhancer-binding protein β
**CE** – cholesterol ester
**Cer** – ceramide
**CERK** – ceramide kinase
**CerS** – ceramide synthase
**CERT** – ceramide transfer protein
**ChIP** – chromatin immunoprecipitation
**coIP** – co-immunoprecipitation
**CoRNR** – corepressor nuclear receptor box
**CREB** – cAMP responsive element binding protein
**CYP** – nomenclature of genes encoding P450 monoxygenase enzyme
**DAX-1** – dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (NR0B1)
**DGK** – diacylglycerol kinase
**DHEA** – dehydroepiandrosterone
**DHEAS** – DHEA sulfate
**E$_2$** – 17β-estradiol
**EGF** – epithelial growth factor
**ER** – estrogen receptor
**ER** – endoplasmic reticulum
**ERE** – estrogen receptor response element
**ERK** – extracellular signal regulated kinase
**FAPP2** – four-phosphate adaptor protein 2
**GCN5 –** general control nonderepressor 5
**GLUT4** – glucose transporter 4
**GM** – ganglioside
**GPCR** – G-protein coupled receptor
**GPER/GPR30** – G-protein coupled estrogen receptor 1
**GR** – glucocorticoid receptor
**HDAC** – histone deacetylase
**HDL** – high-density lipoprotein
**HexCer** – hexosylceramide
**hCG** – human chorionic gonadotropin
**HSD** – hydroxysteroid dehydrogenase
**HSL** – hormone sensitive lipase
**Hsp90** – heat shock protein 90
**IGF-I** – insulin-like growth factor 1
**IL-1β** – interleukin 1 β
**INF-γ** – interferon γ
**JKN** – c-Jun N-terminal kinase
**KLF6** – Kruppel-like factor 6
**KSR** – kinase suppressor of Ras
**LacCer** – lactosylceramide
**LAMP1** – lysosome-associated membrane protein 1
**LBD** – ligand binding domain
**LDL** – low-density lipoprotein
**LDLR** – low-density lipoprotein receptor
**LH** – leutenizing hormone
**LRH-1**- liver receptor homolog 1 (NR5A2)
**MAPK** – mitogen-activated protein kinase
**Mc2R** – melanocortin 2 receptor
**MNAR** – modulator of non-genomic activity of estrogen receptor
**NCoR1** – nuclear receptor corepressor1
**NE** – nuclear envelope
**NF-κB** – nuclear factor κB
**NGF** – nerve growth factor

**NGFI-B/Nur77** – nerve-growth factor BI (NR4A1)
**NOE** – N-oleoylethanolamine
**Nor-1** – neuron-derived orphan receptor 1 (NR4A3)
**Nurr1** – nuclear receptor related 1 (NR4A2)
**PA** – phosphatidic acid
**PCNA** – proliferating cell nuclear antigen
**PCOS** – polycystic ovary syndrome
**PDGF** – platelet-derived growth factor
**PE** – phosphatidylethanolamine
**PGC-1α** – PPARγ coactivator α
**$PGE_2$** – prostaglandin E2
**PI3K** – phosphoinositide-3-kinase
**PIP** – Phosphatidyl inositol phosphate
**PKA** – protein kinase A
**PKC** – protein kinase C
**PLC** – phospholipase C
**PLD** – phospholipase D
**PMA –** phorbol 12-myristate 13-acetate
**PP1** – protein phosphatase 1
**PP2A** – protein phosphatase 2A
**PPARγ** – peroxisome proliferator-activated receptor γ
**PR** – progesterone receptor
**PTX** – pertussis toxin
**Rb** – retinoblastoma gene product
**re-ChIP** – sequential ChIP
**RNAi** – RNA interference
**S1P** – sphingosine-1-phosphate
**S1PR** – sphingosine-1-phosphate receptor
**SAP** – saposin
**SAPK** – stress-activated protein kinase
**SF-1** – steroidogenic factor-1 (NR5A1)
**SGPP** – S1P phosphatase
**Shc** – Src-homology 2 domian containing protein
**SHP** – small heterodimer partner
**shRNA** – short hairpin RNA
**siRNA** – small-interfering RNA
**SPHK** – sphingosine kinase
**SM** – sphingomyelin
**SMase** - sphingomyelinase
**SMRT** – silencing mediator for retinoid and thyroid-hormone receptors
**SMSr** – SM-synthase-related protein
**Sp** – specificity protein
**SPH** – sphingosine
**SPL** – S1P lyase
**SPT** – serine palmitoyltransferase
**SPTLC2** – SPT long chain subunit 2
**SR-BI** – scavenger receptor class B type I
**SRC-1** – steroid receptor coactivator 1
**SRC-2** – steroid receptor coactivator 2
**SREBP** – sterol regulatory element-binding protein
**STAT** – signal transducer and activator of transcription
**StAR** – steroidogenic acute regulatory protein
**TNF-α** – tumor necrosis factor α
**TORC2** – target of rapamycin complex 2
**TSPO** – 18-kDa translocator protein
**WT** – wild type

## SUMMARY

Steroid hormones, such as cortisol and aldosterone, are synthesized from cholesterol through a series of enzymatic reactions in steroidogenic-competent tissues. In the human adrenal cortex, cortisol biosynthesis is controlled by adrenocorticotropin (ACTH) via the activation of a cAMP-dependent signaling pathway. ACTH signaling induces the transcription of all the genes required for steroid hormone production. The nuclear receptor steroidogenic factor 1 (SF-1) is the primary transcriptional regulator of most steroidogenic genes. Our laboratory has uncovered a link between ACTH signaling and sphingolipid metabolism by demonstrating that ACTH rapidly activates sphingolipid turnover concomitant with sphingosine-1-phosphate (S1P) secretion from H295R human adrenocortical cells, and identifying sphingosine (SPH) as an antagonist for SF-1. Sphingolipids are bioactive molecules that modulate multiple cellular functions. We also demonstrated that SPH-dependent repression of SF-1 function is dependent on the expression of acid ceramidase (ASAH1), an enzyme that forms SPH by hydrolyzing ceramide (Cer). Based on these data, I hypothesized that ACTH/cAMP signaling regulates ASAH1 function at both transcriptional and post-transcriptional levels. In addition, because SF-1 is predominantly a nuclear protein, I postulated that ASAH1 modulates SF-1 function and, therefore, steroidogenic gene expression by controlling the nuclear concentrations of SPH. To test these hypotheses, I first examined the effect of chronic ACTH/cAMP signaling on the transcription of the ASAH1 gene. Next, the functional significance of ASAH1 expression in adrenocortical cells was probed by generating an ASAH1-knockdown cell line. I subsequently characterized the role of ASAH1 as a transcriptional nuclear receptor coregulator. Finally, I defined the role of S1P, a bi-product of ASAH1 activity, in the acute phase of cortisol biosynthesis. Using a variety of experimental approaches, I identified cAMP response element binding protein (CREB) as an essential transcriptional activator of the ASAH1 gene. Analysis of adrenocortical cells lacking ASAH1 revealed that ASAH1 is a global regulator of steroidogenic capacity. Furthermore, I identified ASAH1 as a nuclear protein and defined the molecular determinants of the interaction between ASAH1 and SF-1. Collectively, this body of work establishes the integral role of ASAH1 in the regulation of ACTH-dependent adrenocortical cortisol biosynthesis.

# INTRODUCTION

## 1.1. Steroid hormone biosynthesis in the human adrenal cortex

Adrenal steroid hormones, such as cortisol and aldosterone, are important endocrine messengers that are involved in a vast array of physiological processes including glucose metabolism, inflammation, and electrolyte and fluid balance (1-4). The vital role that these molecules play in human physiology dictates the need for a complex network of regulatory mechanisms that act concertedly to maintain optimal circulating plasma hormone concentrations. All steroid hormones are derived from cholesterol through a series of enzymatic reactions, which is defined as steroidogenesis (i.e. the production of steroids). Cholesterol-metabolizing enzymes and other accessory proteins are selectively expressed in different steroidogenic tissues to assure the production of steroid hormones in a tissue-specific manner.

The adrenal gland is comprised of a neuroendocrine medulla and a steroidogenic cortex. The adrenal cortex, which makes up most of the adrenal gland, can be sub-divided into 3 distinct zones, each with a characteristic steroidogenic profile: (1) the zona glomerulosa is the outer cortical zone where the mineralcorticoid aldosterone is produced, (2) The middle zone, zona fasciculata, makes glucocorticoids (e.g. cortisol), and (3) the inner zone, zona reticularis, is the site of androgen (DHEA and its sulfate DHEA-S, and androstenedione) biosynthesis. Each of the three cortical zones expresses a unique profile of steroidogenic enzymes, thereby allowing for zone-specific cholesterol metabolism (5-6) (Figure 1.1)

In the zona fasciculata and reticularis, activation of the steroid hormone biosynthetic pathway is initiated when the peptide trophic hormone ACTH binds to the melanocortin 2 receptor (Mc2R), a cell surface G-protein coupled receptor (GPCR). Hormone binding activates two temporally distinct responses: a rapid phase and a slower chronic response. In the acute phase, stored cholesterol esters are cleaved and

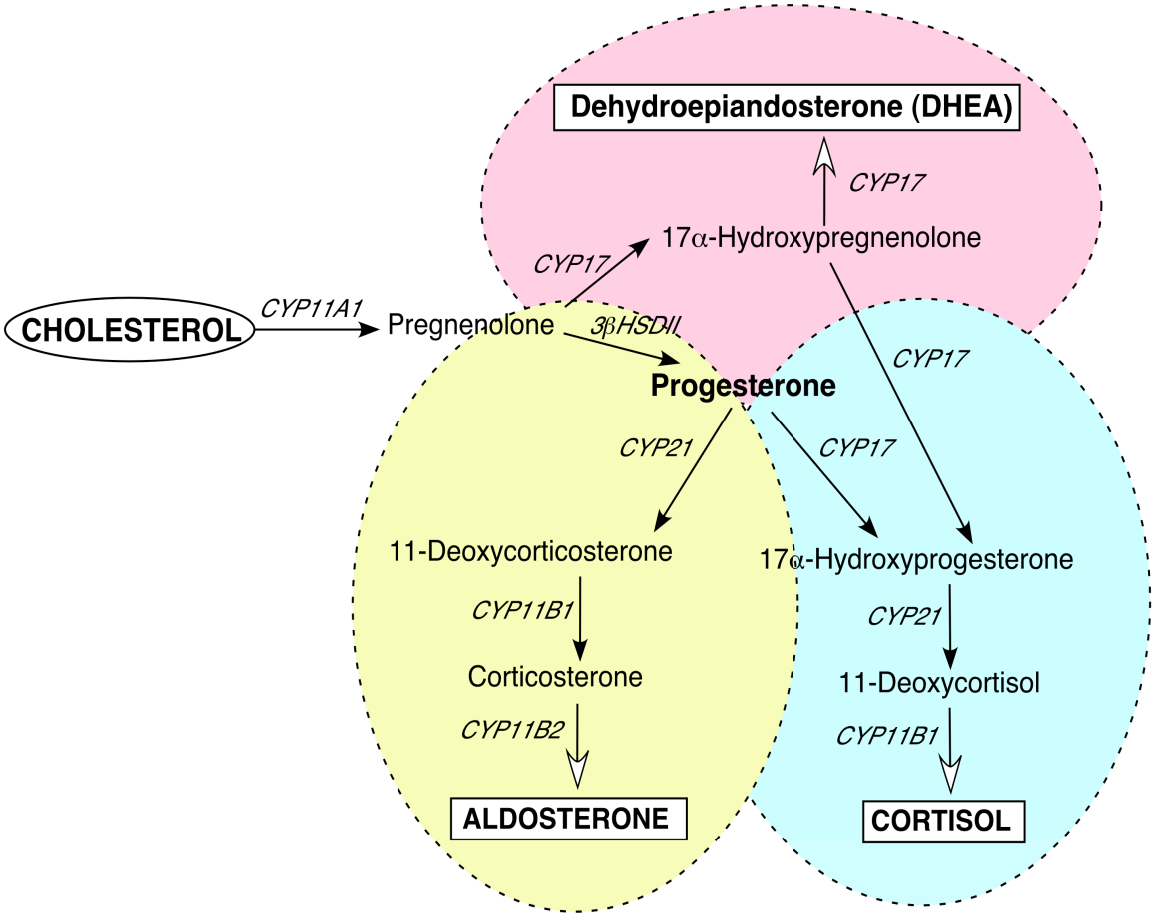



**Figure 1.1.** *Steroid hormone biosynthetic pathway and steroidogenic genes of the three zones of the adrenal cortex*. Genes that encode P450 or hydroxysteroid dehydrogenase enzymes responsible for each catalytic step in cholesterol metabolism are depicted in *italics*. The three steroidogenic zones are distinguished by different colors: zona fasciculata (blue), zona reticularis (pink), and zona glomerulosa (yellow).

transported to the inner mitochondrial membrane, the site of the first enzymatic step of cholesterol metabolism. Hormone sensitive lipase (HSL) (7) catalyzes the de-esterification of newly imported and stored cholesterol esters, a pre-requisite for its utilization in steroid hormone production. Several transport proteins then facilitate the transport of free cholesterol into mitochondria, the rate-limiting step in steroid hormone production (Figure 1.2). One key protein is steroidogenic acute regulatory protein (StAR) (8-11), the founding member of the START (StAR-related lipid transport) family of transport proteins. StAR is rapidly transcribed, translated, and localized to mitochondria

upon hormonal stimulation (12-14). In addition to StAR, 18-kDa translocator protein (TSPO) (15), PKA regulatory subunit-Iα (PKA-RIα)- associated protein, and voltage-dependent anion channel (VDAC) (12,16-17) form a macromolecular complex that facilitated cholesterol mobilization into the inner mitochondrial membrane.

In the chronic phase of steroidogenesis, all the genes responsible for cholesterol metabolism are transcriptionally activated (Figure 1.2). The activation of ACTH signaling culminates in the interaction of various transcription factors, including GATA4 and GATA6 (18-20), CREB (21-22), specificity protein (Sp) family members (18,23), nerve growth factor 1B (NGF-1B, NR4A1) (24-25), and SF-1 (26-27), with promoters of target steroidogenic genes. A detailed description of SF-1 function is presented in Section 1.3 of this Introduction. At this point, it is sufficient to note that because SF-1 plays a major role in integrating extracellular signals (i.e. ACTH) and gene expression, elucidating the mechanisms that controls receptor activity is central for the complete understanding of ACTH actions in steroidogenic cells. As it will be discussed throughout subsequent introductory Sections, the present work defines the role of ASAH1, the enzyme that generates the SF-1 ligand SPH, as a regulator of receptor function and cortisol production in adrenocortical cells.

### 1.2. The Cortisol Biosynthetic Pathway

Cortisol is the primary human glucocorticoid and regulates inflammation (1), carbohydrate and lipid metabolism, and the stress response (28). In target tissues, cortisol mediates its effects via activation of the glucocorticoid receptor (GR) and, in some tissues, the mineralcorticoid receptor, both of which regulate the transcription of several genes (29-30). As stated previously, the biosynthesis of this steroid hormone is primarily regulated by ACTH via the activation of a cAMP/PKA-dependent signaling pathway. Two classes of steroidogenic enzymes are responsible for cholesterol

metabolism: the cytochrome P450 heme-containing proteins (CYPs) and hydroxysteroid dehydrogenase (HSD) enzymes (27,31). P450 side chain cleavage (P450scc) (encoded by CYP11A1) is an inner mitochondria membrane-bound enzyme that catalyzes the first enzymatic reaction in the synthesis of all steroid hormones: cleavage of free cholesterol into pregnenolone (Figure 1.1). P450c17$\alpha$ (17$\alpha$-hydroxylase/17,20-lyase) is an endoplasmic reticulum (ER)-localized enzyme encoded by the CYP17A1 gene. P450c17$\alpha$ catalyzes the hydroxylation of progesterone and pregnenolone at the carbon-17 as well as the conversion of pregnenolone to DHEA in the zona reticularis and the conversion of progesterone into androstenedione in the zona fasciculata. The microsomal 3$\beta$-hydroxysteroid dehydrogenase type II (3$\beta$-HSDII) catalyzes the conversion of pregnenolone, 17$\alpha$-hydroxypregnenolone, and DHEA into progesterone, 17$\alpha$-hydroxyprogesterone, and androstenedione, respectively. P450c21 hydroxylase, encoded by CYP21A2, is also microsomal and catalyzes the conversion of progesterone and 17$\alpha$-hydroxyprogesterone into 11-deoxycorticosterone and 11-deoxycortisol, respectively. P45011-$\beta$-hydroxylase (CYP11B1) is localized in the inner mitochondrial membrane and converts 11-deoxycorticosterone or 11-deoxycortisol into corticosterone or cortisol in the zona fasciculata. In the zona glomerulosa, aldosterone synthase (CYP11B2) is expressed in the inner mitochondrial membrane and catalyzes the conversion of 11-deoxycorticosterone into aldosterone (Figure 1.1).

The zone-specific expression of CYP11B1, CYP17A1 (31), and cytochrome b5, an electron transfer protein that potentiates P450c17$\alpha$ activity (32-36), allow for glucocorticoid production in the zona fasciculata and androgen synthesis in the zona reticularis of the adrenal cortex. Increased 17,20-lyase activity of P450c17$\alpha$ drives androgen synthesis in the zona reticularis. Conversely, the absence of CYP17A1 and presence of CYP11B2 in the zona glomerulosa allows for mineralocorticoid secretion.

Temporal expression of these enzymes is also important for proper steroid hormone production during development.

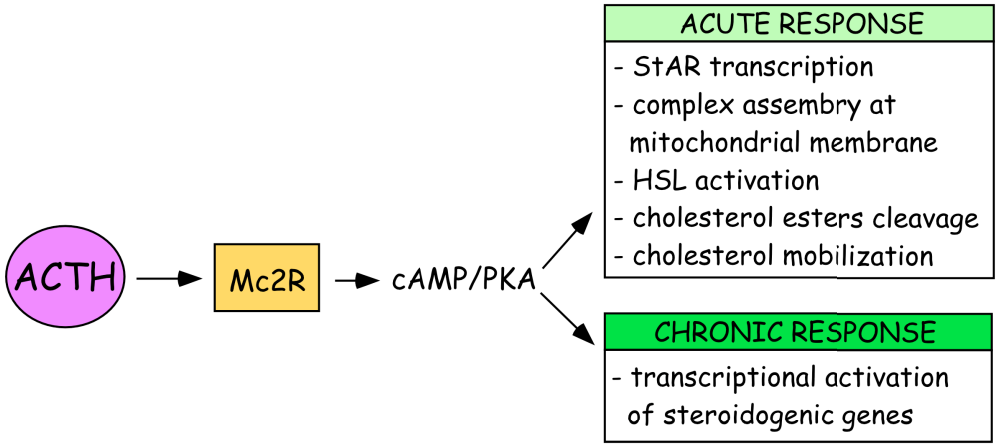



**Figure 1.2.** *ACTH activates two temporally distinct responses in target cells.* Adrenocorticotropin (ACTH) binds to the melanocortin 2 receptor (Mc2R) at the cell surface of target cells and activates a cAMP/protein kinase A (PKA)-dependent pathway. Activation of this pathway rapidly (acute response) upregulates the activity of hormone sensitive lipase (HSL), which cleaves cholesterol esters into free cholesterol, and steroidogenic acute regulatory protein (StAR), which facilitates cholesterol transport into the inner mitochondrial membrane. Chronically, ACTH/cAMP signaling induces the transcription of all the genes required for cholesterol transport and metabolism into steroid hormone.

### 1.3. Sphingolipids and the sphingolipid metabolic pathway

Sphingolipids are a large family of glycolipids and phospholipids that share a common sphingoid base backbone (Figure 1.3), which is often derivatized with an amide-linked fatty acid to make Cer (N-acylsphingosine) and other head groups and/or substituents to form more structurally complex sphingolipid molecules. These lipids are not only important structural components of cell membranes but also act as second messengers in various signaling transduction pathways (37-51). Aberrant sphingolipid metabolism is linked to various disease states including insulin resistance (52-54), cancer (39,55-56), and neurodegeneration (57-59), thus illustrating their importance in the maintenance of proper cellular functions. The structural diversity of this family of lipids is vast and many intermediate metabolites are highly bioactive. Therefore,

sphingolipid metabolism is tightly regulated by a series of metabolizing enzymes that work concomitantly to regulate sphingolipid homeostasis and signaling (Figure 1.4).

De *novo* sphingolipid biosynthesis begins with the condensation of L-serine and palmitoyl-CoA by serine palmitoyltransferase (SPT) to form 3-ketodihydrosphingosine. This intermediate is rapidly metabolized to dihydrosphingosine (sphinganine) and dihydroceramide by the catalytic activities of 3-ketosphinganine reductase and Cer synthase (CerS), respectively (Figure 1.4). Six mammalian CerS isoforms have been described, each utilizing fatty acyl-coAs with varying chain lengths and thus producing Cer subspecies with different fatty acyl-chain compositions (60). Recent lipidomic analysis revealed that Cer with varying fatty acyl-chain length have distinct cellular functions (61-62), which exemplifies the importance of multiple CerS isoforms. Desaturation of dihydroceramide forms Cer, which constitutes the basic structure of higher order sphingolipids (Figure 1.3). Distinct complex sphingolipid species is formed by the combination of different head groups to Cer {e.g. O-linked phosphocholine and carbohydrates to form sphingomyelin (SM) or glycosphingolipids [e.g. hexosylceramide (HexCer) and lactosylceramide (LacCer)], respectively}. Additional sphingolipid moieties with varying backbone and fatty-acyl chain length, number and position of double bonds, and hydroxyl and methyl group substituents have also been described (63). Ceramidases catalyze the breakdown of Cer to form SPH, which can be phosphorylated by sphingosine kinases (SPHK1 and SPHK2) to generate S1P. Alternatively, Cer can be phosphorylated by ceramide kinase (CERK) to form ceramide-1-phosphate (C1P). S1P can be irreversibly metabolized into hexadecenal and phosphoethanolamine by S1P lyase (SPL) or recycled back to SPH by S1P phosphatases (SGPP1 and SGPP2) (Figure 1.4).

Recently, it was shown that SPT is able to utilize L-alanine (64), L-glycine (64), and shorter acyl-CoA molecules (65-66) as alternative substrates, thus generating

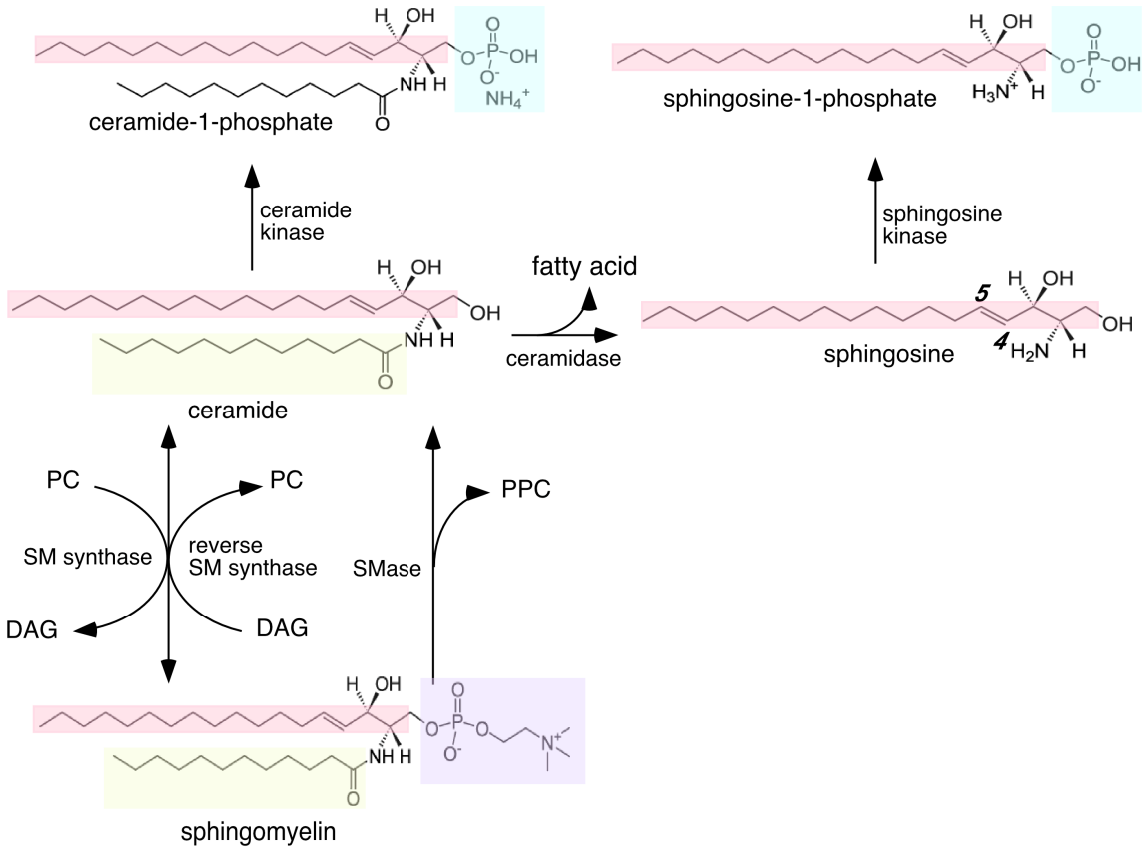



**Figure 1.3.** *Chemical structure of selected sphingolipid species and their metabolism.* Numbers '4' and '5' on the structure of sphingosine designates the position of the double bond. Diffferent chemical groups are highlighted by different colors: Sphingoid backbone (pink), phosphate group (blue), phosphorylcholine (purple), N-acyl-chain (yellow). *Abbreviations*: phosphatidylcholine (PC), phosphorylcholine (PPC), diacylglycerol (DAG), sphingomyelin (SM), sphingomyelinase (SMase).

atypical metabolites and expanding the list of possible physiologically important sphingolipid species. There are a multitude of physiological and cellular roles for individual sphingolipid species, including SM, Cer, C1P, SPH, and S1P (38,40,43-44,46-47,54,67-80). SM is the most abundant sphingolipid in mammalian cells and, in addition to being an important membrane component, is the primary intracellular source of Cer (Figure 1.4). As will be discussed later, many extracellular factors induce intracellular Cer accumulation by activating sphingomyelinase (SMase), the enzyme responsible for the degradation of SM into Cer (Figure 1.3). Cer participates as a second messenger in numerous cellular events including apoptosis, senescence, and cell cycle arrest (81-83)

while its phosphorylated form, C1P, promotes cell differentiation and survival (68,84-85). Similar to Cer and C1P, SPH and S1P have opposing roles in cellular processes: the former acts as a pro-apoptotic agent (67,86-87) while the later mediates cell migration, proliferation, and survival (40,42,49,88). Regulatory roles for SPH and S1P in adrenocortical steroidogenesis will be discussed in subsequent Sections of this Introduction. Defining their roles in the regulation of steroidogenic gene expression is one of the aims of this dissertation.

Because different sphingolipids have specific cellular functions, the intracellular concentrations of different sphingolipid species are tightly controlled by sphingolipid metabolizing enzymes. Most of these enzymes are localized to specific subcellular compartments, where they act to maintain sphingolipid homeostasis in distinct microenvironments (89). Enzymes linked to *de novo* sphingolipid synthesis (e.g. SPT, CerS, dihydroceramide desaturase, Figure 1.4) are primarily localized in the ER lumen whereas enzymes associated with the production of more complex sphingolipids (e.g. SM synthase and glucosylceramide synthase, Figure 1.4) reside in the Golgi apparatus. The mechanism by which sphingolipids are transported between two cellular compartments (e.g ER-Golgi, different Golgi stacks, and cytosolic side-lumen side of an organelle) during *de novo* biosynthesis is an important issue. To date, two sphingolipid-transfer proteins have been identified: CERT (Cer transfer protein) and FAPP2 (four-phosphate adaptor protein 2). CERT mediates ER-to-Golgi non-vesicular transport of Cer for SM synthesis (90-91). FAPP2, on the other hand, transfer glucosylceramide to appropriate Golgi sites for the synthesis of complex glycosphingolipids (92). Enzymes responsible for sphingolipid catabolism, such as different isoforms of ceramidase and SMase, are expressed in the plasma membrane, lysosomes, and mitochondria (93). Because sphingolipids are mainly hydrophobic, the subcellular location where these molecules are generated typically dictates their site of action. Determining the

intracellular localization of ASAH1 in adrenocortical cells is one of the questions addressed in the present work.

### 1.4. Acid Ceramidase: a key regulator of ceramide metabolism

Ceramidases (*N*-acylsphingosine amidohydrolase) are lipid hydrolases that catalyze the degradation of Cer into SPH and a free fatty acid (Figure 1.3). In vertebrate cells, five human ceramidase genes have been cloned and their encoded enzymes are categorized by the pH at which they exhibit optimal *in vitro* activity as acid (ASAH1), neutral (ASAH2), and three isoforms of alkaline (ACER1-3) (37). Each isoform has a different subcellular localization (94-99) and substrate specificity (96,100-103), which suggest that these enzymes have unique biological functions. Furthermore, the tissue distribution and expression levels of each isoform differ. ASAH1 and ACER3 are ubiquitously expressed and have high mRNA abundance whereas ACER1 expression is high in skin (37) (Table 1.1). ASAH2 and ACER2 are also ubiquitously expressed but have a low mRNA abundance (37), suggesting that post-trascriptional mechanisms are involved in their regulation (Table 1.1). Because Cer degradation is the only source of cellular SPH (104), these enzymes are not only essential for limiting Cer-mediated signaling but also for controlling the cellular functions of SPH and its phosphorylated form, S1P (83,105-106).

ASAH1 is a glycoprotein processed from a 55 kDa precursor into a 14 kDa $\alpha$ and a 40 kDa $\beta$ subunits via autoproteolytic cleavage (107-108). This enzyme has been reported to localize to lysosomes (99) and to be secreted extracellularly from murine endothelial cells, macrophages, and human fibroblasts (98). ASAH1 not only function to regulate sphingolipid metabolism but also participate in signal transduction pathways to regulate various physiological and pathological processes. In mouse, ASAH1 is expressed early during embryogenesis, with targeted disruption of the ASAH1 gene

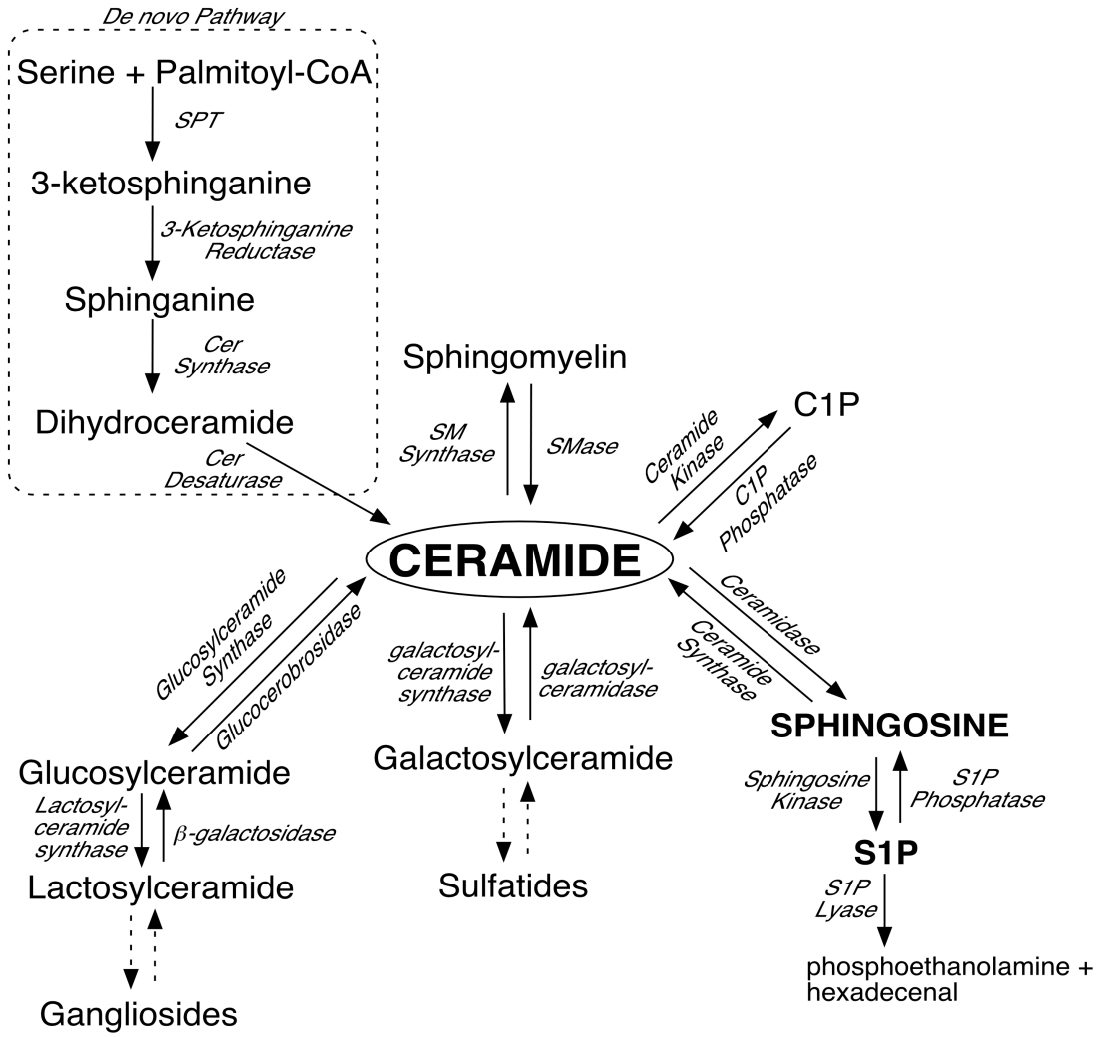



**Figure 1.4.** *The sphingolipid metabolic pathway. Abbreviations*: serine palmitoyltransferase (SPT), ceramide (Cer), sphingosine-1-phosphate (S1P), sphingomyelin (SM), sphingomyelinase (SMase), ceramide-1-phosphate (C1P).

resulting in embryonic lethality (109). Moreover, ASAH1 overexpression has been reported in various human cancers (110-113) and a genetic deficiency in ASAH1 catalytic activity causes the lysosomal sphingolipid storage disorder, Farber's disease (114). Therefore, although the precise role of ASAH1 in the etiology of these conditions is unclear, it is likely that the function of this enzyme as a regulator of the intracellular levels of Cer, SPH, and S1P is crucial for cellular homeostasis.

As stated previously, our laboratory has demonstrated that ACTH signaling promotes rapid changes in sphingolipid concentrations, including SM and Cer turnover

and the secretion of S1P from H295R cells (75). S1P was characterized as an activator of CYP17A1 transcription (115) while its unphosphorylated counterpart, SPH, was identified as an antagonist for SF-1 (47). The intricate details of these findings are discussed in later Sections of this Introduction. At this point, what can be appreciated is the potential role of ASAH1 in maintaining the capacity of the human adrenal gland to regulate cortisol secretion, and thus the inflammatory response and gluconeogenesis. As a SPH-generating enzyme, ASAH1 plays a pivotal role in regulating the intracellular concentrations of SPH and S1P. Therefore, defining the significance of ASAH1 expression in ACTH-stimulated adrenocortical steroidogenesis and elucidating the functional roles of this ceramidase in the regulation of SF-1 transcriptional activity are two of the central goals of this dissertation.

**Table1.1.** *Summary of the current knowledge about protein sequences, tissue distributions, substrate specificity, and cellular localizations of the different ceramidase isoforms*. *Abbreviations:* acid (ASAH1), neutral (ASAH2), and alkaline (ACER) ceramidase, plasma membrane (PM), golgi apparatus (GA). Under 'substrate specificity', N-acyl chain length and number of double bonds are denoted as x:y, respectively. Table adapted from Mao *et al.* 2008 (37).

| ***Gene Symbol*** | ***Cellular localization*** | ***pH optimum*** | ***Substrate specificity*** | ***mRNA abundance*** |
|---|---|---|---|---|
| ASAH1 | lysosome | 4.5 | C6- to C16-Cer | ubiquitous (high) |
| ASAH2 | PM | 7.0 | ≥ C14-Cer | ubiquitous (low) |
| ACER1 | ER | 8.5 | ≥ C24:1-Cer | High in skin |
| ACER2 | GA | 9.0 | ≥ C14-Cer | ubiquitous (low) |
| ACER3 | ER/GA | 9.5 | ≤ C20:1-Cer | ubiquitous (high) |

### 1.5. Steroidogenic Factor 1 (SF-1): a key transcription factor in adrenal steroidogenesis

The nuclear receptor SF-1 (Ad4BP/NR5A1) plays an essential role in the development and function of steroidogenic tissues by controlling the expression of most steroidogenic genes. In the human adrenal cortex, SF-1 is recruited to the promoter of target genes in response to ACTH/cAMP signaling. Because of the central role of this receptor as a master regulator of steroidogenic gene transcription, uncovering the

molecular underpinnings of receptor function is essential for understanding ACTH action. SF-1 has a unique tissue distribution within steroidogenic tissues, being expressed in all three zones of the adrenal cortex, Sertoli and Leydig cells in the testis, granulosa, theca, and corpus luteum cells in the ovary, as well as in the placenta, and adipose tissue (116). SF-1 is a member of the nuclear receptor superfamily of transcription factors (117) and has a modular domain structure: an amino-terminal conserved DNA binding domain (DBD) consisting of two zinc-binding modules, an intervening hinge region containing a ligand-independent activation domain (AF1), and a carboxy-terminal ligand-binding domain (LBD) containing a conserved AF-2 hexamer domain (LLIEML) that forms a helical structure (denoted as 'helix 12') and is critical for receptor activation (118) (Figure 1.5). The zinc-finger module has a central role in recognizing the canonical AGGTCA DNA sequence recognized by all nuclear receptors. The hinge region and LBD participate in transcriptional repression or ligand-dependent activation. These domains serve as the interface for interactions between SF-1 and numerous coregulatory proteins, including steroid receptor coactivator 1 (SRC-1, NC0A1) (119-120), SRC-2 (GRIP1, NC0A2) (121), nuclear receptor corepressor 1 (NCoR1) (122), NCoR2/SMRT (silencing mediator for retinoid and thyroid hormone receptors) (121), and GCN5 (general control nonderepressed 5, KAT2A) (123).

In adrenocortical cells, SF-1 is an integral regulator of StAR (22,124), Mc2R (125), CYP17A1 (126-127), CYP11B1 (128), CYP21A2 (129), 3β-HSDII (130), and CYP11A1 (131-132) gene transcription. SF-1 is also essential for gonadal and adrenal development (133-134) as evidenced by the phenotype of targeted disruption of the receptor in mice (135-136). Strong evidence for a developmental role of SF-1 originates from studies of forced SF-1 expression in various SF-1-negative cell lines. Studies of embryonic stem cells and mesenchymal cells from mice and human bone marrow revealed that SF-1 expression induces the expression of multiple steroidogenic genes

and promotes steroid hormone synthesis (137-139). Similarly, a recent study has highlighted a novel role for SF-1 in stem cell differentiation into gonadal steroid-producing cells (140). The central role of this receptor in steroidogenesis is also evident in humans where mutations in the receptor result in various clinical pathologies including gonadal dysgenesis, adrenal failure, sex reversal, and underandrogenization (141). Furthermore, mice studies using tissue-specific conditional knockouts of SF-1 revealed novel roles for this receptor in modulating neural function (142-144). Similarly, SF-1 has been implicated in the regulation of energy homeostasis (145-146).

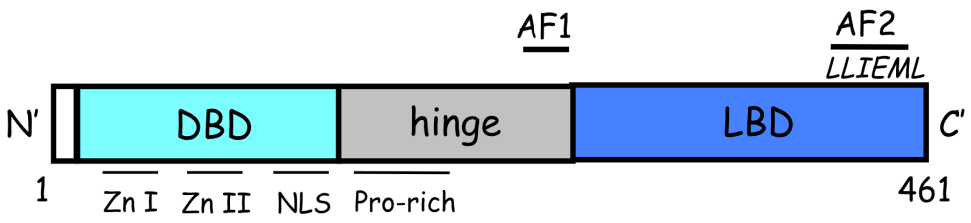



**Figure 1.5.** *Schematic overview of SF-1.* SF-1 contains the major structures of all nuclear receptors: the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD). The DBD contains two zinc-fingers (Zn I, Zn II) and the nuclear localization signal (NLS) while the hinge has a proline-rich region (Pro-rich). *Abbreviations:* Activation function domains 1 (AF1) and 2 (AF2). Numbers at the bottom of the diagram indicate residue number.

Most nuclear receptors are cytoplasmic and translocate into the nucleus in response to ligand binding and dimerization. Differently, SF-1 is a predominantly nuclear protein that binds as a monomer (147) to target gene promoters that contain at least one element of C/TCAAGGTCA (121). Its transcriptional function is regulated by various post-translational modifications including phosphorylation (148-151), sumoylation (152-153), acetylation (154-156), as well as by protein-protein interactions (126,157-162). More recently, ligand binding has been implicated in the regulation of SF-1 activity (155,163-167). Crystallographic studies using bacterially expressed SF-1 demonstrated that phospholipids are present in the receptor's ligand binding pocket (163,165,167). As stated previously, our laboratory has identified SPH and the phospholipid phosphatidic

acid (PA) as endogenous ligands for SF-1 (164,166). SPH acts as an antagonist for SF-1 whereas PA enhances receptor activity. SPH binds to SF-1 under basal conditions and cAMP promotes its displacement from the receptor ligand-binding pocket (Figure 1.6). Furthermore, our group has found that diacylglycerol kinase θ (DGKθ), the enzyme that produces PA, interacts with SF-1 in the nucleus of H295R cells. We postulated that an interaction between these two proteins facilitate ligand delivery to the ligand-binding pocket of SF-1 (Figure 1.6). Based on these findings, I hypothesized that a similar interaction occurs between ASAH1 and SF-1. Because SPH has low water solubility, direct binding of ASAH1 to the receptor would facilitate ligand exchange. Multiple experimental approaches will be used to address this question.

### *1.5.1. SF-1 coregulators*

Transcriptional activation by nuclear receptors requires dynamic protein-protein interactions between the receptor, coregulator proteins, and the RNA polymerase II transcription machinery at target gene promoters. Coregulators are not only sufficient to promote nuclear receptor activity but also required for optimal receptor activation. These regulatory factors either have intrinsic catalytic properties or recruit enzymatic protein complexes to mediate chromatin modifications that influence gene transcription. Coregulators act to activate (i.e. coactivators) or repress (i.e. corepressors) transcription. Coactivators generally enhance transactivation of agonist-bound nuclear receptor by interacting with the receptor through at least one LXXLL motif (also called NR boxes), where X is any amino acid and L is a leucine (168). Importantly, modified NR boxes where the leucine residues are substituted with isoleucine (I), phenylalanine (F), or methionine (M) have also been described (169-170). On the receptor side, formation of a hydrophobic cleft in the LBD and AF1 and/or AF2, as a result of ligand binding, is usually

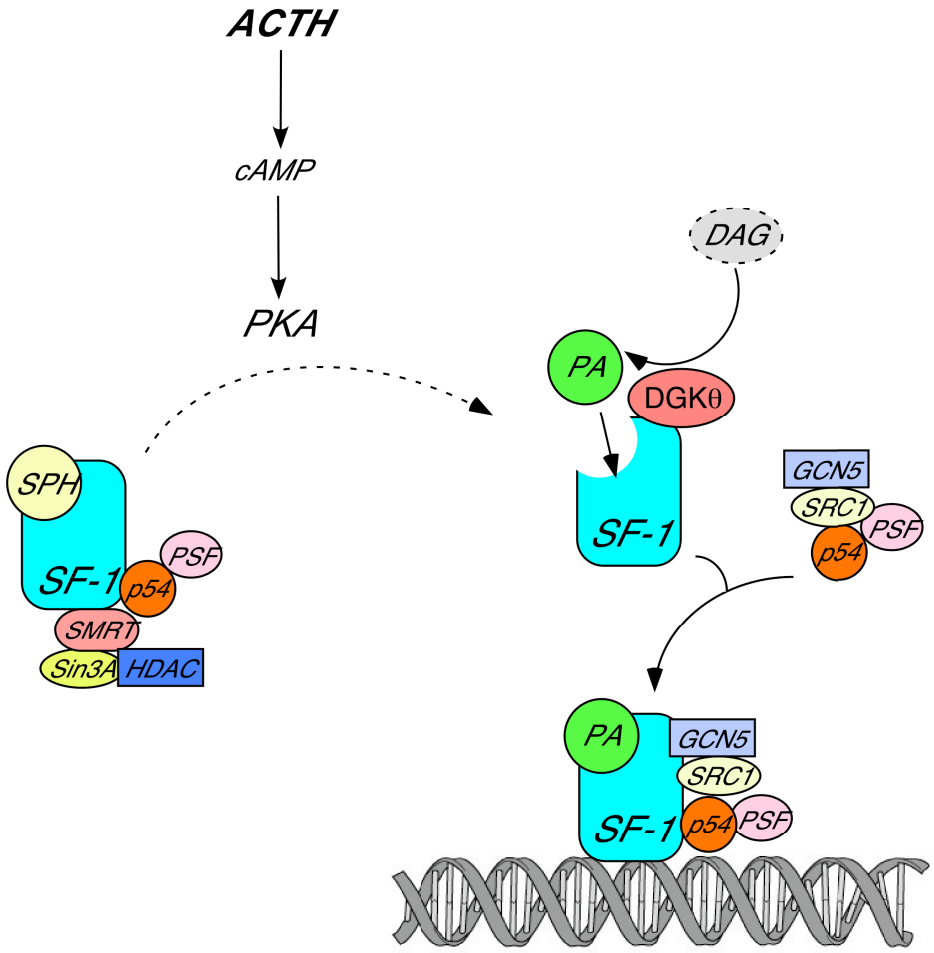



**Figure 1.6.** *Model for the role of ligands in controlling steroidogenic factor 1 (SF-1) transactivation potential.* Under basal conditions, sphingosine (SPH) is bound to SF-1 and corepressors. ACTH signaling activates protein kinase A (PKA), which promotes the release of SPH from the receptor's ligand binding pocket. Concomitantly, activation of the ACTH/cAMP pathway increases nuclear diacylglycerol kinase θ (DGKθ) activity, leading to increased phosphatidic acid (PA) biosynthesis. PA binding to SF-1 activates the receptor and facilitates its recruitment to target gene promoters and association with coactivator proteins. *Abbreviations:* silencing mediator for retinoid and thyroid hormone receptors (SMRT), steroid receptor coactivator 1 (SRC1), general control nonderepressed 5 (GCN5), histone deacetylase (HDAC), polypyrimidine tract-binding protein-associated splicing factor (PSF).

required for interaction with coactivators (171). The p160 family of coactivators, which include SRC-1, SRC-2, and SRC-3 (ACTR/RAC-3, NC0A3), are among the most studied coregulators (172).

Corepressors, on the other hand, are characterized by selectively repressing nuclear receptor activity by interacting with unliganded or antagonist-bound receptor. Usually, these proteins have repression domains that serve as docking platforms for the recruitment of enzymes associated with transcriptional suppression, including histone deacetylases (HDAC). NCoR1 and SMRT were the first corepressors to be identified. They were found to interact with nuclear receptors through helical motifs denoted CoRNR (corepressor nuclear receptor) box with consensus sequence (L/I)-XX-(I/V)-I or L-XXX-(I/L)-XXX-(I/L) (168). Subsequently, additional corepressors that were able to interact with agonist-bound nuclear receptors, such as RIP140 (receptor interacting protein 140, NRIP1) and LCoR (ligand-dependent nuclear receptor corepressor), were identified. Most of these corepressors have LXXLL motifs (similar to coactivators) that mediate binding to the AF-2 domain of nuclear receptors (173). However, opposite to coactivators, these LXXLL-containing corepressors recruit corepressor protein complexes to suppress gene transcription. The ability of either class of coregulators to bind to nuclear receptors is determined by the ligand-dependent positioning of helix 12 (AF-2) relative to the LBD. Ligand binding triggers the movement of helix 12 towards the LBD, increasing steric accessibility of the binding surface, thus facilitating coactivator binding (174). Conversely, in the absence of ligand, helix 12 adopts an extended conformation that removes steric restrains and allows corepressor binding (173).

As stated above, SF-1 has been shown to interact with many coregulators including SRC-1 (119-120), SRC-2 (121), NCoR1 (122), SMRT ((121), and GCN5 (123). Using temporal chromatin immunoprecipitation (ChIP) in synchronized H295R cells, our laboratory has characterized the components of protein complexes that associate with

the CYP17A1 promoter during cAMP-stimulated transcriptional activation (158). Early in the transcriptional cycle, cAMP promotes the formation of a complex containing SF-1, SRC-1, and GCN5 on the CYP17A1 promoter. Dissociation of this complex is followed by the recruitment of HDAC1, RIP140, and the corepressor Sin3A. Subsequently, another cycle of transcription is enabled by the re-association of SF-1 and RNA polymerase II on the promoter (158). Building upon these findings, some of the experiments described in this dissertation will explore the function of ASAH1 in controlling SF-1 activity and the role of coregulators as potential mediators and/or disruptors of this process.

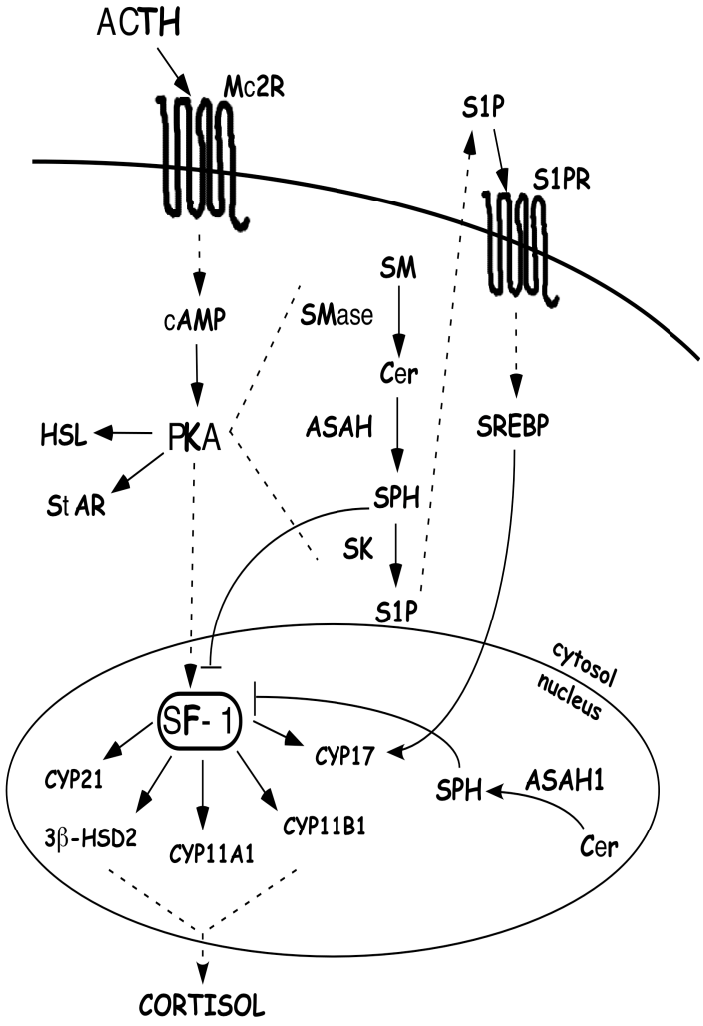



**Figure 1.7.** *Model pathway of the crosstalk between ACTH/PKA signaling and sphingolipid metabolism in the human adrenal cortex.* ACTH binding to melanocortin 2 receptor (Mc2R) activates cAMP production and protein kinase A (PKA) activation, which stimulates sphingolipid metabolism, activates hormone sensitive lipase (HSL) and steroidogenic acute regulatory protein (StAR) as well as SF-1. SF-1 then induces the transcription of all steroidogenic genes required for cortisol production. Sphingosine (SPH) produced in the cytosol and nucleus antagonizes SF-1 function while sphingosine-1-phosphate (S1P) enhances CYP17A1 transcription via a receptor-mediated activation of steroid regulatory element binding protein (SREBP). *Abbreviations*: Ceramidase (ASAH), acid ceramidase (ASAH1), sphingomyelinase (SMase), sphingosine kinase (SK).

## 1.6. Sphingolipid signaling in steroidogenesis

A growing body of literature has established the integral role that distinct sphingolipid species play in steroid hormone production. One of the mechanisms by which extracellular signals (e.g. ACTH) control steroidogenesis is by modulating the intracellular concentrations of sphingolipids. For example, as previously stated, ACTH rapidly activates sphingolipid metabolism in H295R cells by decreasing the intracellular amounts of SM, Cer, and SPH, while concomitantly increasing S1P secretion via SPHK activation (75). S1P, in turn, acts as a paracrine/autocrine activator of CYP17A1 transcription (115) (Figure 1.7). Therefore, there is mounting evidence to support the interconnection between sphingolipid metabolism and gene-specific regulation of SF-1 targets. Generally, sphingolipids act at different levels of the steroidogenic signaling pathway, including (I) functioning as paracrine/autocrine regulators, (II) participating in regulatory signaling cascades as second messengers, and (III) serving as ligands for nuclear receptors.

### *1.6.1. Sphingolipids as paracrine/autocrine regulators*

As mentioned previously, S1P is formed by the phosphorylation of SPH (Figure 1.4). This bioactive lipid acts intracellularly as a second messenger or extracellularly by binding and activating signaling through cell surface S1P receptors (S1PRs) in a paracrine and/or autocrine manner. In steroidogenesis, the regulatory functions of S1P are primarily mediated through S1PRs. Our laboratory has demonstrated that S1P is secreted from cAMP-stimulated H295R cells and induces CYP17A1 transcription via a paracrine mechanism that requires S1PR activation and nuclear translocation of sterol regulatory element binding protein 1 (SREBP1) (115) (Figure 1.7). Similarly, in the zona fasciculata of bovine adrenal cells, S1P stimulates cortisol biosynthesis by activating protein kinase C (PKC) and phospholipase D (PLD) through a pertussis toxin (PTX)-

sensitive receptor-mediated mechanism (175). Furthermore, S1P has been implicated in the production of aldosterone in the zona glomerulosa of bovine adrenal cells through an $S1PR_{1/3}$-mediated mechanism involving the activation of phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal regulated kinase (ERK) pathways (176). These findings provide compelling evidence for the integral role of S1P in controlling steroid hormone biosynthesis.

S1P is generated intracellularly by the actions of SPHK1 and SPHK2 (Figure 1.4), with both having similar endogenous substrate specificities, i.e. SPH and dihydroSPH (177-179), although SPHK2 is also able to phosphorylate the immunosupressant synthetic sphingoid base analog FTY720 (fingolimod) (180). In mammals, these isoenzymes have a wide and overlapping tissue distribution, with SPHK1 being highly expressed in spleen and lung, and SPHK2 being predominantly expressed in brain, liver, and heart (181-182). Homozygous single knockout mice of either SPHK1 or SPHK2 are viable and display no obvious phenotypes (183-184). However, the apparent functional redundancy between these isoforms may not always apply, specially under a variety of pathophysiological states (185). SPHK2 overexpression leads to growth arrest and cell death (186-187) whereas SPHK1 has been demonstrated by numerous functional studies to play a role in protecting cells from apoptosis and promoting cell growth and differentiation (188-189). The disparity of physiological effects between these isoenzymes may be partially due to their opposing effects on Cer levels. SPHK2 expression enhances the metabolism of S1P back to Cer (Figure 1.4) and, therefore, leads to Cer accumulation (190). Conversely, SPHK1 expression decreases Cer levels, partially due to inhibition of CerS (191). Additionally, *in vitro* assays have demonstrated that SPHK1 and SPHK2 are differentially regulated (182). From a comparison standpoint, much more is known about the physiological roles of SPHK1 than SPHK2. SPHK1 is considered the major enzyme responsible for S1P

synthesis and it is regulated at all stages of expression (i.e. transcriptional and post-translational regulatory mechanisms).

The movement of intracellular S1P to the extracellular millieu is thought to occur through specific transporters of the ATP-binding cassette (ABC) family of transporters, including ABCC1, ABCA1, and ABCG1 (192-195). However, explicit physiologic and genetic evidence of the precise mechanisms by which these transporters export S1P are yet to be defined. Extracellularly, S1P is bound to high-density lipoproteins and other plasma membranes (e.g. albumin), which facilitate S1P delivery to S1PRs on the surface of target cells (196).

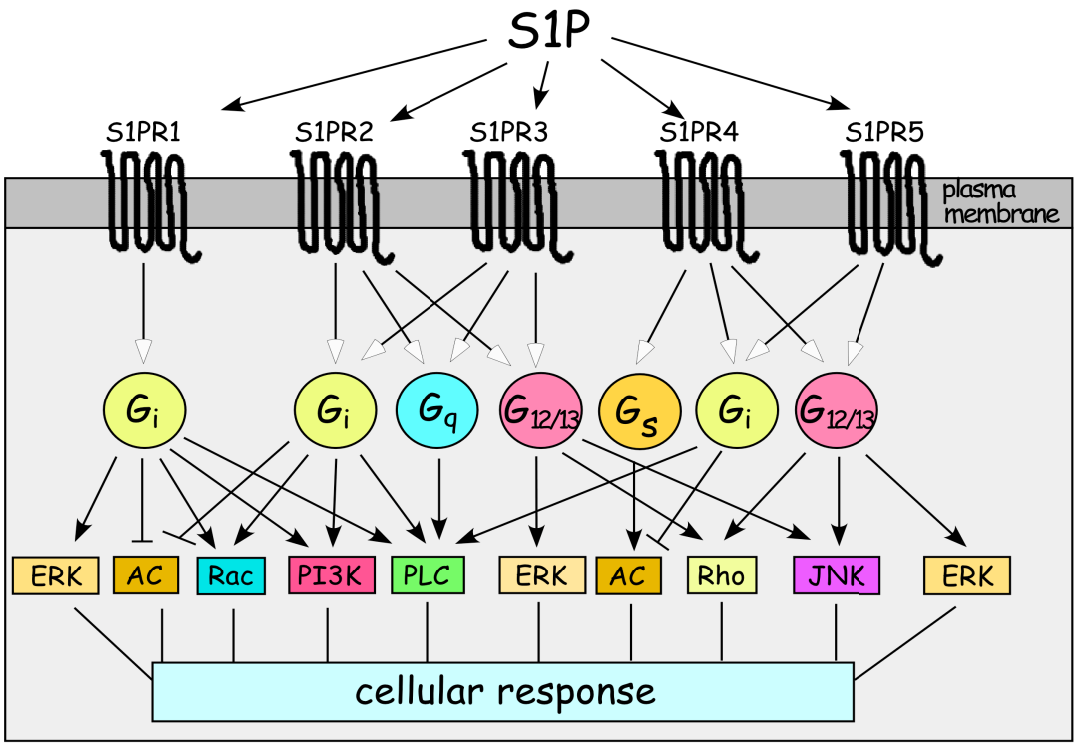



**Figure 1.8.** *S1P receptors (S1PR) and their G protein coupling and downstream signaling pathways. Abbreviations*: extracellular signal regulated kinase (ERK), adenylyl cyclase (AC), phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), c-Jun $NH_2$-terminal kinase (JNK).

There are 5 S1PRs, four of which ($S1PR_{1-3}$ and $S1PR_5$) are expressed in H295R cells (115). Each receptor couples to multiple heterotrimeric G proteins (197-199)

(Figure 1.8) and significant work has been done to characterize the downstream signaling cascades associated with the activation of the different S1PRs. $S1PR_1$ couples to $G_i$ and activates the phospholipase C (PLC), PI3K, and ERK pathways. $S1PR_2$ and $S1PR_3$ couple to $G_i$, $G_{12/13}$, and $G_q$ and active multiple downstream cascades including PLC, PI3K, ERK, c-Jun $NH_2$-terminal kinase (JNK), and Rho GTPase. $S1PR_4$ activates PLC and ERK whereas $S1PR_5$ inhibits adenylyl cyclase and ERK while activates JNK (reviewed in (197,200)) (Figure 1.8). As previously stated, work from our group established S1P as an inducer of CYP17A1 gene expression in H295R cells (Figure 1.7). Given the multitude of possible signaling pathways activated by S1PRs (Figure 1.8) and the role of ceramidases in controlling S1P synthesis (Figure 1.4), some of the experiments described in this thesis are aimed at determining the role of S1P signaling during the acute phase of steroidogenesis (Section 1.1).

### *1.6.2. Sphingolipids as second messengers in steroidogenic regulatory pathways*

Arguably, the most extensively characterized regulator of steroidogenesis is Cer, which accumulates in steroidogenic cells in response to various extracellular regulatory signals, including cytokines and growth factors (201-207). In most cases, Cer accumulates as a result of activation of SM hydrolysis (203,208-209). Ultimately, increased Cer levels alter cellular steroidogenic output. Given that Cer comprises the structural backbone for all sphingolipids (Figure 1.3), Cer can modulate steroid hormone production both directly and indirectly through metabolism into other bioactive sphingolipids (Figure 1.4). Importantly, because Cer is the substrate for ceramidases, ASAH1 plays a pivotal role in controlling the signaling properties of Cer.

Cer has been shown to regulate progesterone and testosterone production. In ovarian granulosa cells, interleukin-1β (IL-1β)-mediated activation of SMase suppresses

progesterone production in a Cer-dependent manner (207). In Leydig cells, SM hydrolysis and Cer accumulation have been implicated in reduced progesterone (202) and testosterone (210) secretion through suppression of StAR protein expression. Notably, Cer accumulation observed in these reports was triggered by tumor necrosis factor $\alpha$ (TNF-$\alpha$) signaling, which, similar to IL-1$\beta$**,** activates SMase activity (211). Similarly, TNF-$\alpha$ signaling represses the activity of P450 aromatase (CYP19A1), the enzyme that converts testosterone into estradiol, in granulosa cells through a mechanism involving Cer production (206). Furthermore, Cer suppresses human chorionic gonadotropin (hCG)-stimulated testosterone and progesterone production in a dose-dependent manner (205,212). Finally, Cer was shown to modulate the mRNA expression of 11$\beta$-hydroxysteroid dehydrogenase type I (11$\beta$-HSDI), the glucocorticoid reactivation enzyme, in pre-adipocytes (201). Cell-permeable $C_2$-Cer (N-acetoyl-D-*erythro*-sphingosine) induces the expression and recruitment of CCAAT/enhancer binding protein $\beta$ (C/EBP$\beta$) to the 11$\beta$-HSDI gene. In addition, Cer treatment upregulates 11$\beta$-HSDI activity in these cells (201), thus suggesting a role for Cer in regulating the reactivation of circulating steroid hormones in addition to their *de novo* synthesis.

Although most studies have demonstrated a role for Cer as an inhibitor of steroidogenesis, other reports present opposing findings. Soboloff *et al.* (213) reported that different acyl-chain length Cer have opposite effects on luteinizing hormone (LH)-induced progesterone production in hen granulosa cells. $C_6$-Cer (N-hexanoyl-D-*erythro*-sphingosine) and $C_8$-Cer (N-octanoyl-D-*eythro*-sphingosine) increase intracellular [$Ca^{2+}$] and progesterone secretion whereas $C_2$-Cer has no effect on intracellular $Ca^{2+}$ and suppresses steroid hormone production. Further, Kwun *et al.* (214) demonstrated that $C_2$-Cer increases basal and hCG-stimulated progesterone production in MA-10 testicular Leydig cells. Because the nature of the fatty acyl-chain in Cer was recently found to be

important in dictating the cellular functions of Cer (213,215-220), discrepancies among different published studies are likely due to the use of Cer with different acyl-chain lengths.

Most of the reported mechanisms by which extracellular factors regulate sphingolipid concentrations are post-transcriptional, i.e. regulation of enzyme activity. However, extracellular signals can also trigger changes in sphingolipid content by inducing the transcription of genes encoding sphingolipid-metabolizing enzymes. For example, neural growth factor (NGF) was shown to induce transcription of the SPHK1 gene via binding of Sp1 to a specific 5' region of the promoter (221). Similarly, the nuclear factor - κB (NF-κB) upregulate SGPP2 gene transcription in response to inflammatory stimuli (222) and all-trans retinoic acid (RA) inhibits expression of the CERK gene in neuroblastoma cells (223). Nonetheless, the regulatory mechanisms controlling the expression of most steroidogenic genes, including ASAH1, remains to be determined. In order to determine if ACTH/cAMP signaling regulates ASAH1 at the transcriptional level, some of the experiments described in this dissertation are centered at the functional characterization of the ASAH1 promoter in ACTH/cAMP-stimulated H295R cells.

### *1.6.3. Sphingolipids as ligands for nuclear receptors*

As discussed previously, our laboratory has contributed to expanding the role of sphingolipids in steroidogenesis by demonstrating that SPH is an antagonist for SF-1 (47). SPH is bound to the receptor under basal conditions and exchanged for PA, an SF-1-agonist (164), upon cAMP stimulation (Figure 1.6). SPH antagonizes cAMP-stimulated CYP17A1 reporter gene activity and antagonizes coactivator recruitment. Importantly, silencing ASAH1 expression mimics cAMP-induced CYP17A1 transcription, further supporting the role of SPH as a repressor of SF-1 activity (47) (Figure 1.6). Interestingly,

we have also found that lysosphingomyelin (sphingosylphosphorylcholine) is able to bind to SF-1 under basal conditions and that cAMP treatment promotes its dissociation from the receptor (47). The implications of this binding have not been defined but it suggests that multiple sphingolipids can potentially regulate receptor activity.

Ligand binding adds yet another level of regulation to SF-1 function. To have a comprehensive understanding of the molecular mechanisms governing SF-1-dependent gene expression, the interplay between dynamic post-translational modifications (Section 1.5) and ligand binding needs to be explored. Moreover, the precise molecular mechanism by which SF-1 ligand availability is controlled is unknown. In the present work, various experimental approaches are used to address the role of ASAH1 in controlling the intracellular concentrations of SPH. Based on our previous findings indicating that SPH-dependent suppression of CYP17A1 transcription is dependent on ASAH1 expression (47), I hypothesized that ASAH1 controls SPH availability, and therefore, modulates SF-1 function in the human adrenal cortex.

### 1.7. Nuclear localization of ASAH1 as a pre-requisite for its proposed regulatory function in adrenocortical cells

As mentioned earlier, SF-1 differs from the majority of nuclear receptors in that its activation neither involves cytoplasm to nucleus translocation nor dimerization. Rather, SF-1 is a predominant nuclear protein that binds as a monomer to the promoter of target genes in response to hormone stimulation (Section 1.5). As a result, the nuclear localization of ASAH1 may be a pre-requisite for its hypothetic function as a regulator of SF-1 activity. Given the hydrophobic nature of most sphingolipids, the localized (i.e. nuclear) production of SPH, may be essential for its ability to act as an SF-1 antagonist. Therefore, I postulated that ASAH1 regulates SF-1 function by interacting with the receptor in the nucleus of H295R cells.

### *1.7.1. Nucleus organization and endonuclear domains*

The nucleus is a well-organized sub-structure with a dynamic framework (224). It is composed of a well-defined nuclear envelope (NE) that encapsulates several endonuclear domains, including the nuclear matrix, chromatin, and nucleolus (Figure 1.9). The NE is a bilayer whose outer and inner leaflets display unique lipid compositions. Although a detailed comparison of the relative distribution of lipid species between the two leaflets of the nuclear membrane has not been reported, cholesterol has been shown to reside in the outer but not the inner membrane (225) whereas the gangliosides GM1 and GD1a were detected in both (226). The outer membrane is continuous with the ER and thus shares certain lipidomic properties. Conversely, the inner membrane is closely associated with the nuclear lamina and has distinct lipid characteristics (224) (Figure 1.9).

Like plasma membranes, nuclear membranes express many types of receptors, including inositol 1,4,5-triphosphate ($IP_3$) and RA receptors (227). Some agonists activate signaling exclusively through nuclear membrane-localized receptors. RA, for example, has been shown to activate phospholipase A2 (PLA2), PLC, and PLD only in the nucleus (228). Additionally, compelling new evidence suggest that nuclear membrane-associated enzymes have different physicochemical properties than their plasma membrane and/or cytosolic counterparts. For example, the kinetic parameters of nuclear PLC differ from PLC associated with the plasma membrane or cytoplasm (229).

The nuclear matrix is often viewed as the basic organizing structure of the nucleus (Figure 1.9) that is responsible for maintaining nuclear shape and where many processes, including DNA replication, gene transcription, and protein phosphorylation, take place (230-232). For example, many enzymes linked to phosphatidyl inositol phosphate (PIP) metabolism associate with the nuclear matrix (233), suggesting that the

matrix is actively involved in nuclear lipid signaling cascades. Chromatin is closely associated with the nuclear matrix and exhibits a dynamic structure that is actively modified by multiple interconnected mechanisms, including DNA methylation and histone modification (234-235). Heterochromatin regions, which are transcriptionally inactive but contain many specific nuclear proteins that regulate gene transcription (224), are similarly organized by the nuclear matrix (Figure 1.9).

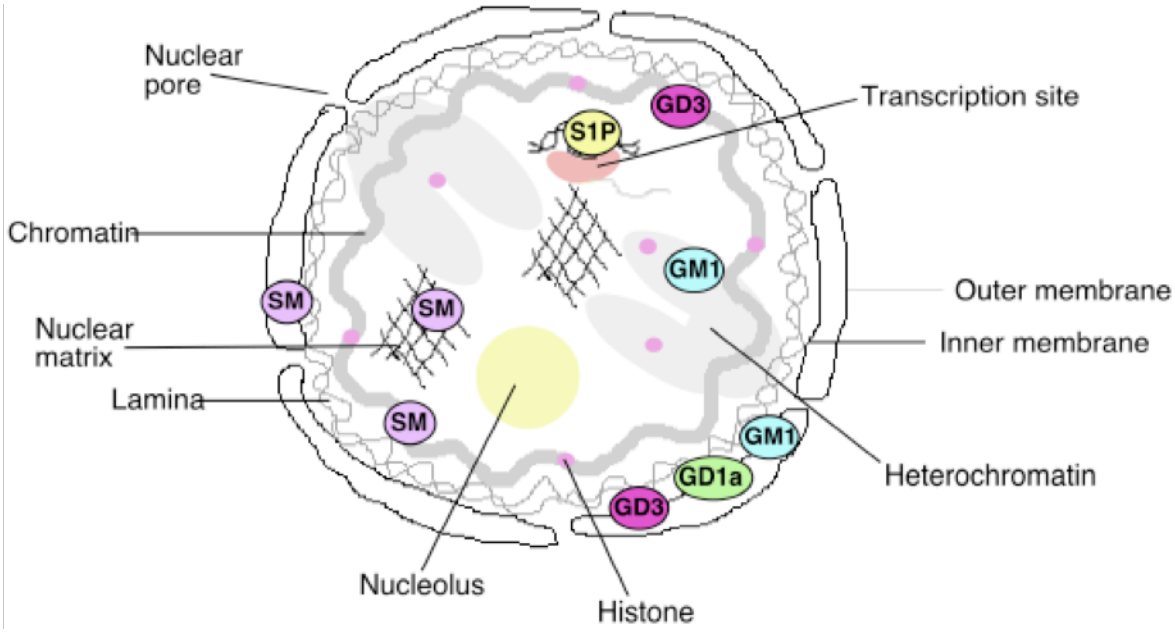



**Figure 1.9.** *Representation of the nucleus and subnuclear domains highlighting the localization of different sphingolipid species.* The outer membrane is continuous with the endoplasmic reticulum while the inner membrane is associated with the nuclear lamina. Nuclear pore allows passive flow of small molecules between cytosol and nucleoplasm. *Abbreviations:* Sphingomyelin (SM), monosialotetrahexosylganglioside (GM1), disialotetrahexosylganglioside (GD1a), sphingosine-1-phosphate (S1P).

### *1.7.2. Nuclear Sphingolipid Metabolism*

Recent studies have uncovered the extensive metabolism of lipids that occurs in the nuclei of various cell types (236-237). These studies have pointed towards important signaling and regulatory roles for lipids in numerous nuclear processes, including DNA replication, RNA processing, chromatin structure, and $Ca^{2+}$ homeostasis (reviewed in

(236-239)). Most lipids are localized to the NE, where in addition to providing structural support, they participate in multiple signaling cascades. Bioactive lipids are also localized in other nuclear compartments including chromatin (240-242) and the nuclear matrix (243) (Figure 1.9). The concentration of nuclear lipids can be dynamically altered by metabolic flux in response to signaling cascades that can often uncouple from cytosolic processes (236-239). PIPs are the most extensively characterized nuclear lipids and have pivotal roles in chromatin remodeling, gene transcription, and mRNA export (163,244-245). However, the nucleus is emerging as a hub for sphingolipid metabolism as well.

Biochemical, analytical, and microscopic techniques have been utilized to identify sphingolipid-metabolizing enzymes in nuclei and to quantify the concentrations of sphingolipid species (246). To date, multiple sphingolipid enzymes have been detected in the nuclei of various cell types. SMase was reported in the nuclear matrix (243,247-248), NE (249), and chromatin (247) whereas SM synthase, the enzyme that catalyzes the formation of SM from Cer (Figure 1.3), was detected in chromatin and NE (250). In addition, nuclear ceramidase and SPHK activities were demonstrated in rat hepatocytes and Swiss 3T3 cells, respectively (251-253). SPHK2 is the predominantly nuclear isoform of SPHK in many cells (254). This SPHK isoform was recently shown to modulate gene expression by regulating histone acetylation (255). Due to the hydrophobic nature of most sphingolipids, the nuclear expression of sphingolipid enzymes suggest that there is a dynamic and localized production of bioactive lipids, which may have unique roles in nuclear processes that are independent from their cytoplasmic functions.

SM is the most prominent sphingolipid in the nuclei, with its concentration in nuclear matrix being three times higher than in chromatin (256). SM is a major component of chromatin (241) (Figure 1.9) where it plays a role in DNA replication and

chromatin architecture (249,257-258). Similarly, glycosphingolipids have been shown to promote cytoprotection through regulation of nuclear $Ca^{2+}$(259). The ganglioside GM1 forms a complex with a $Na^{+}/Ca^{2+}$ exchanger in the NE and facilitates the transfer of $Ca^{2+}$ from the nucleoplasm to the ER lumen (260). Nuclear Cer has been shown to participate in Fas-induced apoptosis in Jurkat T cells as a result of caspase-3-dependent activation of SMase (261). Recently, Hait *et al.* (255) linked nuclear SPHK2/S1P to epigenetic regulation of gene expression by demonstrating that SPHK2 is associated with HDAC1/2 in repressor complexes. S1P inhibits HDAC1/2 activity and induces $p21^{CIP1/WAF1}$ and C-FOS gene transcription by enhancing histone H3 acetylation (255). Collectively, studies of nuclear sphingolipids not only illustrate the multifaceted regulatory capabilities of these lipid mediators, which, in most cases, differ from their cytoplasmic functions, but also highlight the importance of location (e.g. chromatin versus NE versus nuclear matrix) in determining their nuclear functions.

As stated earlier, SPH not only serves as the precursor of S1P but also plays an important regulatory role in the nucleus of steroidogenic cells by acting as an antagonist for SF-1 (47) (Figure 1.6). In this manner, SPH regulates gene expression and steroid hormone secretion (47) (Figure 1.7). SF-1 is a nuclear protein and, as discussed above, nuclear expression of sphingolipid enzymes allow for localized and dynamic sphingolipid metabolism within various endonuclear compartments. Therefore, the nuclear localization of ASAH1 is likely to be imperative for my hypothesis of ASAH1 controlling SF-1 function to hold true. This is a central question that will be addressed in this dissertation. Furthermore, building upon this question, the physical interaction between these proteins will be investigated and the role of various SF-1 coregulators (Section 1.5.1) in bridging ASAH1 function to SF-1 activity will be addressed. Collectively, these experiments form the basis for testing the central hypothesis of the present work, which

is that ASAH1 modulates SF-1 function and steroidogenic gene expression by controlling the nuclear concentrations of SPH.

## CHAPTER 2:

*Sphingosine-1-phosphate rapidly increases cortisol biosynthesis and the expression of genes involved in cholesterol uptake and transport in H295R adrenocortical cells*

### 2.1. Introduction

In the zona fasciculata of the human adrenal cortex, cortisol is synthesized from cholesterol via the concerted action of P450 heme-containing monooxygenases and 3β-HSD enzymes (262-264). Additionally, StAR, TSPO, HSL, low-density lipoprotein receptor (LDLR), and scavenger receptor class B type I (SR-BI) are equally important in assuring adequate amounts of substrate for steroid hormone production. Cortisol biosynthesis is primarily regulated by the peptide hormone ACTH, which upon binding to Mc2R, activates a cAMP/PKA-dependent pathway. Acutely, ACTH promotes cholesterol uptake and mobilization from intracellular stores to the inner mitochondrial membrane, where the first enzymatic step in steroid hormone production occurs. In the chronic phase of steroidogenesis, ACTH coordinately activates the transcription of all genes in the steroid hormone biosynthetic pathway, thus maintaining optimal steroidogenic capacity (265-267).

Import of free cholesterol into the inner mitochondrial membrane is facilitated by the formation of a macromolecular protein complex at the outer mitochondrial membrane containing TSPO, StAR, the TSPO-associated protein PAP7, and the regulatory subunit RIα of PKA (268-269). StAR is essential for cholesterol trafficking (270) and is rapidly synthesized in response to activation of the ACTH signaling pathway (271). The regulation of StAR transcription has been extensively studied (reviewed in (272)), and SF-1 is required for StAR gene expression (273) via its binding to a cAMP-responsive region located within the first 350 base pairs upstream of the transcription initiation site (274-276). Alternatively, $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels abrogates StAR transcription by inducing the expression of the transcription factor DAX-1 (dosage-

sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), a repressor of the StAR gene (277). ACTH/cAMP rapidly increases StAR mRNA and protein levels (278-280) as well as its enzymatic activity through PKA-mediated phosphorylation events at conserved serine residues (281). Further, the PKC signaling pathway also plays an integral role in StAR regulation by potentiating cAMP-stimulated StAR expression and phosphorylation (282). Of note, Manna *et al.* (283) have recently shown that different PKC isoforms, including PKC$\alpha$, PKC$\delta$, PKC$\varepsilon$ and PKD, exhibit diverse effects on phorbol 12-myristate 13-acetate (PMA)-mediated StAR transcription and steroidogenesis. Finally, A-kinase anchoring protein 121 (AKAP121) enhances the post-transcriptional regulation of StAR by recruiting type II PKA regulatory subunit $\alpha$ and StAR mRNA transcripts to the outer mitochondria membrane, thus promoting spatially localized protein synthesis and increasing steroidogenic efficiency (284). Similarly, HSL, which is responsible for the neutral cholesteryl ester hydrolase activity in steroidogenic tissues (285), plays a vital role in steroid hormone production by increasing the availability of free cholesterol. HSL interacts with StAR and this partnering between the two proteins facilitates the transport of cholesterol from lipid droplets to mitochondria (286).

Several studies have reported various roles for sphingolipids in adrenal and gonadal steroidogenesis (287-298). As a bioactive lipid mediator, S1P regulates a broad array of physiological functions, including cell proliferation and survival (299-301), chemotaxis (302-304), and protection against Cer-mediated apoptosis (305). S1P has also been shown to stimulate cortisol production in zona fasciculata bovine adrenal cells in a PKC- and $Ca^{2+}$-dependent manner (306), and promote aldosterone secretion in bovine glomerulosa cells via the PI3K/Akt and MAPK/ERK pathways (290,307). S1P also mediates cAMP-dependent cortisol secretion in H295R cells by promoting the SREBP-1-dependent transcription of CYP17A1 (308).

S1P not only functions as an intracellular messenger (309-310) but also exerts many of its effects through cell surface GPCRs (311). The mechanism of S1P export from cells is not completely understood, however, studies have provided evidence for the involvement of the ABC family of transporters in this process (312-315). Five S1PRs ($S1PR_{1-5}$) have been identified (316-317), four of which ($S1PR_1$, $S1PR_2$, $S1PR_3$, $S1PR_5$) are expressed in H295R steroidogenic cells (308). Differences in signaling through these receptors are primarily due to differential coupling to G-proteins. $S1PR_1$ signals through $G\alpha_i$ (318-319) whereas $S1PR_2$ and $S1PR_3$ couple to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{13}$ (319). $S1PR_4$ associates with $G\alpha_i$ and $G\alpha_{12/13}$ (320-322) and $S1PR_5$ couples to $G\alpha_{i/o}$ and $G\alpha_{12}$ (323).

Our laboratory has shown that ACTH rapidly stimulates sphingolipid metabolism in H295R cells (295). ACTH and the cAMP analog dibutyryl-cAMP ($Bt_2cAMP$) decrease cellular amounts of SM, Cer, and SPH, while simultaneously increasing the secretion of S1P (295). The S1P produced stimulates cortisol secretion from H295R cells by promoting the maturation and binding of SREBP1 to the CYP17A1 promoter, thereby inducing gene transcription (308). These findings implicate S1P as a paracrine mediator of ACTH-dependent CYP17A1 transcription. Therefore, the aim of the present study was to characterize the role of S1P in mediating the acute phase of steroidogenesis in H295R cells. I show that S1P rapidly increases cortisol biosynthesis and the mRNA expression of multiple genes involved in the acute phase of steroid hormone production including StAR, TSPO, SR-BI, and LDLR. In addition, I demonstrate that S1P acutely increases the phosphorylation of HSL at $Ser^{563}$ and show that S1P-stimulated StAR gene expression is PTX-sensitive and dependent on the activation of PLC, CamKII, and ERK1/2.

## 2.2. Materials and Methods

### *2.2.1. Cell culture and Treatment*

H295R adrenocortical cells (324-326) were generously donated by Dr. William E. Rainey (Medical College of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12) (Mediatech, Manassas, VA) supplemented with 10% Nu-Serum I (BD Biosciences, Palo Alto, CA), 1% ITS Plus (BD Biosciences, Palo Alto, CA), antibiotics, and antimycotics. H295R cells were sub-cultured in 12-well plates and pretreated for 18 h with 10 μM VPC23019 ($S1PR_1$ and $S1PR_3$ inhibitor), 5 pg/ml PTX (pertussis toxin), 10 μM U73122 (PLC inhibitor), 10 μM U73343 (inactive PLC analog), 10 μM KN-93 (CamKII inhibitor), or 10 μM U0126 (MEK1 inhibitor) followed by treatment with 1 μM S1P, 50 nM ACTH, or 0.4 mM $Bt_2$cAMP for 2 h.

### *2.2.2. Cortisol and DHEA Assay*

Cells were cultured in 12-well plates and treated with 0.4 mM $Bt_2$cAMP or 1 μM S1P for 3 to 48 h. Cortisol and DHEA released into the media were determined in triplicate against standards made up in DMEM/F12 medium using a 96-well plate enzyme-linked immunosorbent assays (ELISA; Enzo Life Sciences Inc., Plymouth Meeting, PA). Results are expressed as nanomoles per milligram of cellular protein in each sample.

### *2.2.3. RNA isolation and real time RT-PCR (qRT-PCR)*

Total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime, Inc., Gaithersburg, MD) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Scientific Inc., Waltham, MA) and the following primer sets: β-actin (forward 5'-ACG GCT CCG GCA TGT GCA AG-3' and reverse 5'-TGA CGA TGC CGT GCT GCA TG-3'), StAR (forward 5'-GCT CTC TAC TCG GTT CTC-3' and reverse 5'-GCT GAC TCT CCT

TCT TCC-3'), TSPO (forward 5'-GCA GAT TCC GTG ATT ACA GTG-3' and reverse 5'-TCC TCC TCG TCG TCA TCG-3'), HSL (forward 5'-CAC TAC AAA CGC AAC GAG AC-3' and reverse 5'-CCA GAG ACG ATA GCA CTT CC-3'), SR-BI (forward 5'-CCA TCC TCA CTT CCT CAA-3' and reverse 5'-CCA CAG GCT CAA TCT TCC-3', LDLR (forward 5'-ACG GTG GAG ATA GTG ACA ATG-3' and reverse 5'-AGA CGA GGA GCA CGA TGG-3'). mRNA expression of StAR, TSPO, HSL, SR-BI, and LDLR was normalized to the transcript levels of β-actin and calculated using the delta-delta cycle threshold ($\Delta\Delta$CT) method.

### *2.2.4. Measurement of Inositol 1, 4, 5-Trisphosphate ($IP_3$)*

Cells were treated with 1 μM S1P or 0.4 mM $Bt_2$cAMP for 5 to 60 min. Cells were harvested into PBS and centrifuged 5 min at 4,000 rpm. Supernatant was removed and the cells resuspended in 20% (w/v) perchloric acid on ice for 30 min. Cells were centrifuged and the supernatants neutralized with HEPES-KOH solution. Amounts of inositol 1, 4, 5-trisphosphate in each sample were measured by a radioreceptor assay with the D-myo-inositol 1, 4, 5-triphosphate [$^3$H] assay kit (TRK 1000, Amersham Biosciences, Piscataway, NJ).

### *2.2.5. Intracellular $Ca^{2+}$ quantification*

H295R cells were sub-cultured onto coverslips for 24 h and intracellular calcium was labeled with Fluo 3/AM (327-329). Fluo 3/AM (5 mM) was loaded into the cells in serum-free medium for 1 hour at 37 °C. 0.02% pluronic acid (Sigma, St. Louis, MO) was added to the medium to disperse Fluo 3/AM (EMD Biosciences). After the incubation, cells were washed three times with medium. Then cells were placed in a chamber with 0.5 ml medium for fluorescence imaging. Fluorescence images were acquired using

UltraVIEW Vox Spinning Disk Confocal Microscope (Perkin Elmer Inc., Waltham, MA). Intensity ratio of Fluo 3/AM was quantified using the Volocity software (Perkin Elmer Inc.).

### *2.2.6. Western Blotting*

For analysis of StAR protein expression, cells were treated with 1 μM S1P (0 – 18 h). Phospho-ERK1/2 (pERK1/2) and total ERK2 expression was assessed in lysates purified from cells that were serum-starved for 30 h, pretreated with 10 μM KN-93, or 5 pg/ml PTX for 1 h followed by treatment with 1 μM S1P. Phospho-CamKII (pCamKII) and total CamKII expression was assessed in lysates purified from cells that were pretreated with 10 μM VPC23019, 10 μM U0126, or 5 pg/ml PTX for 1 h followed by treatment with 1 μM S1P for 30 min. To assess the phosphorylation status of HSL, cells were treated for 15, 30, or 60 min with 1 μM S1P. Cells were harvested into RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1X Protease Inhibitor Cocktail Set I (EMD Biosciences) and lysed by sonication (one 2 sec burst) followed by incubation on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 15 min at 4° C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (30 μg of protein) were run on 8% SDS-PAGE gels and transferred to Immobilon FL polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were probed with anti-phospho-ERK1/2 (sc-7383, Santa Cruz), ERK2 (sc-154, Santa Cruz), StAR (sc-25806, Santa Cruz), phospho-Ser$^{563}$-HSL (4139, Cell Signaling), HSL (sc-25843, Santa Cruz), phospho- Thr$^{286}$-CamKII (06-881, Millipore), CamKII (04-1079, Millipore), or GAPDH (sc-25778, Santa Cruz). Expression was detected using an ECF western blotting kit (GE Healthcare, Piscataway, NJ) and visualized by scanning the blots on a VersaDoc 4000 imager (Bio-Rad). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

### *2.2.7. Statistical Analysis*

One-way ANOVA and Tukey-Kramer multiple comparison test were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Significant differences from compared value were defined as $p < 0.05$ and denoted by asterisks (*) and carats (^).

## 2.3. Results

### *2.3.1. S1P stimulates cortisol biosynthesis*

As previously discussed, our laboratory has demonstrated that ACTH and $Bt_2$cAMP rapidly stimulate sphingolipid metabolism (295) and S1P secretion from H295R cells (308). Additionally, S1P induces CYP17A1 transcription (308). Therefore, in order to investigate the role of S1P in the acute phase of steroid hormone biosynthesis, I determined the effect of S1P on cortisol biosynthesis in H295R cells. Cells were treated for time points ranging from 3 to 48 h and the concentrations of steroid hormone secreted into the media were quantified as described in Section 2.2. As shown in Figure 2.1A, S1P maximally increased cortisol production by 2.5-fold at the 12-h time point. While S1P resulted in a significant increase in cortisol production at the 48 h time point (Figure 2.1A), this bioactive sphingolipid was unable to evoke a sustained increase in DHEA secretion over the same time period (Figure 2.1B). $Bt_2$cAMP treatment robustly stimulated the secretion of both cortisol and DHEA at all time points assayed, with a 7-

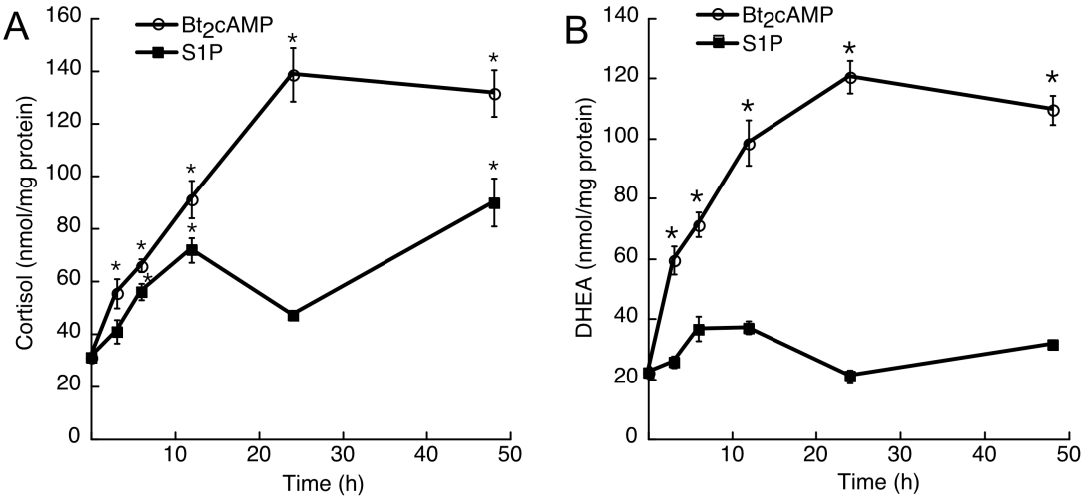



**Figure 2.1.** *S1P increases cortisol secretion.* H295R cells were treated for time periods ranging from 3 to 48 h with $Bt_2$cAMP (0.4 mM) or S1P (1 μM) as described in Section 2.2. Cortisol (**A**) or DHEA (**B**) released into the media was quantified by ELISA. Values represent the mean ± SEM of 3 experiments, each performed in triplicate and normalized to the total cellular protein content. Asterisks (*) denote $p < 0.05$ for agonist-stimulated hormone production versus vehicle-treated controls.

fold increase in cortisol production and a 6-fold increase in DHEA biosynthesis at the 24 h time point.

### *2.3.2. S1P induces the mRNA expression of various acute phase steroidogenic genes*

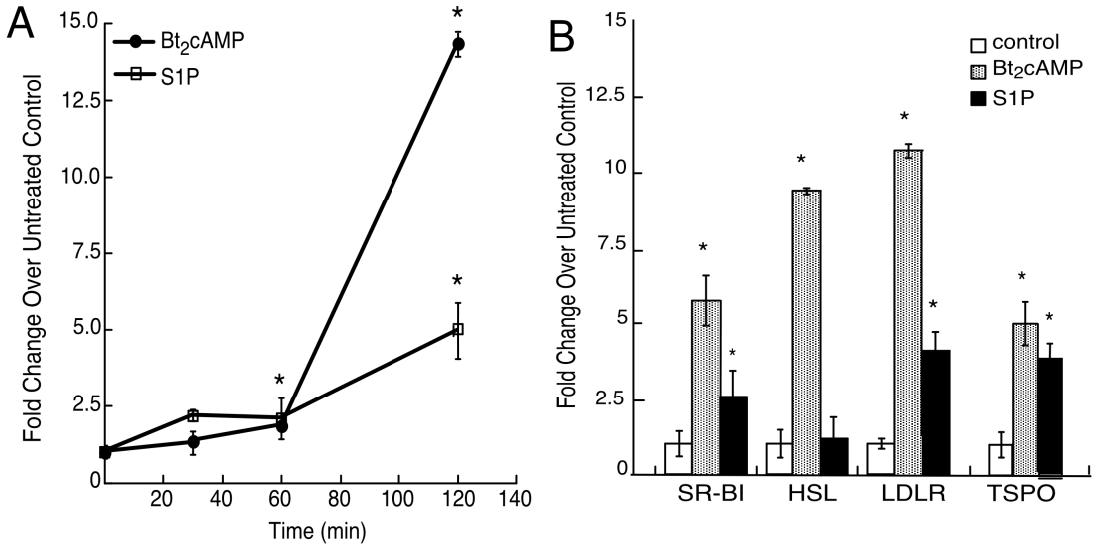



**Figure 2.2**. *S1P acutely increases steroidogenic gene expression*. **A.** H295R cells were treated with 0.4 mM $Bt_2$cAMP or 1 μM S1P for 30 min to 2 h. StAR mRNA levels were quantified by qRT-PCR and normalized to the mRNA expression of β-actin. **B.** H295R cells were treated with 0.4 mM $Bt_2$cAMP or 1 μM S1P for 2 h and mRNA expression of SR-BI, HSL, LDLR, and TSPO was quantified by qRT-PCR and normalized to the mRNA levels of β-actin. Data graphed are expressed as percent of control group mean and represent the mean ± SEM of 3 experiments, each performed in triplicate. Asterisk (*) denotes $p < 0.05$ for $Bt_2$cAMP- or S1P-stimulated mRNA expression versus vehicle-treated control.

To characterize the mechanism by which S1P acutely increases cortisol secretion, the effect of S1P on the mRNA expression of multiple genes involved in the acute steroidogenic response was assessed. StAR is essential for the delivery of free cholesterol into the inner mitochondrial membrane (269-270), the rate-limiting step in steroid hormone biosynthesis. To determine the effect of S1P on the mRNA expression of StAR, H295R cells were treated for 0.5, 1, or 2 h with 0.4 mM $Bt_2$cAMP or 1 μM S1P and total RNA was isolated for quantification by qRT-PCR. As shown in Figure 2.2A,

S1P and $Bt_2$cAMP induced StAR mRNA expression by 2.0- and 1.8-fold after 1 h treatment, respectively. S1P further increased StAR expression at the 2-h time point by 5.0-fold, as compared to the 14-fold induction in StAR mRNA expression elicited by $Bt_2$cAMP (Figure 2.2A).

In addition to examining StAR transcription, the mRNA expression of four additional genes that are involved in the biosynthesis of cortisol was quantified: TSPO, SR-BI, LDLR and HSL. TSPO forms a macromolecular complex with StAR to facilitate the import of cholesterol into the inner mitochondrial membrane (330). SR-BI mediates the selective uptake of cholesteryl esters from high-density lipoprotein (HDL) particles in rodents and humans (331-333) and HSL has neutral cholesteryl ester hydrolase activity and is critical for hydrolyzing internalized cholesteryl esters (334). Importantly, cAMP has been shown to regulate both HSL enzymatic activity and SR-BI mRNA expression (335). LDLR enables receptor-mediated endocytic delivery of cholesteryl esters from low density lipoprotein (LDL) particles (336). In humans, LDLR provides most of the cholesterol necessary for steroid hormone production (337). I examined the mRNA expression of these genes in H295R cells that were treated with 1 μM S1P or 0.4 mM $Bt_2$cAMP for 2 h. As shown in Figure 2.2B, S1P induced the mRNA expression of SR-BI, LDLR, and TSPO by 2.5-, 4.7-, and 4.5-fold, respectively. The mRNA expression of HSL was not affected by S1P, but $Bt_2$cAMP significantly increased the expression of all four genes.

### *2.3.3. S1P-mediated induction of StAR gene expression is pertussis toxin sensitive*

As discussed earlier, many of the effects elicited by S1P are mediated through $S1PR_{1-5}$ that activate multiple downstream signaling cascades (311,317,338). In order to define the signaling pathway that mediates S1P-induced StAR gene expression, the

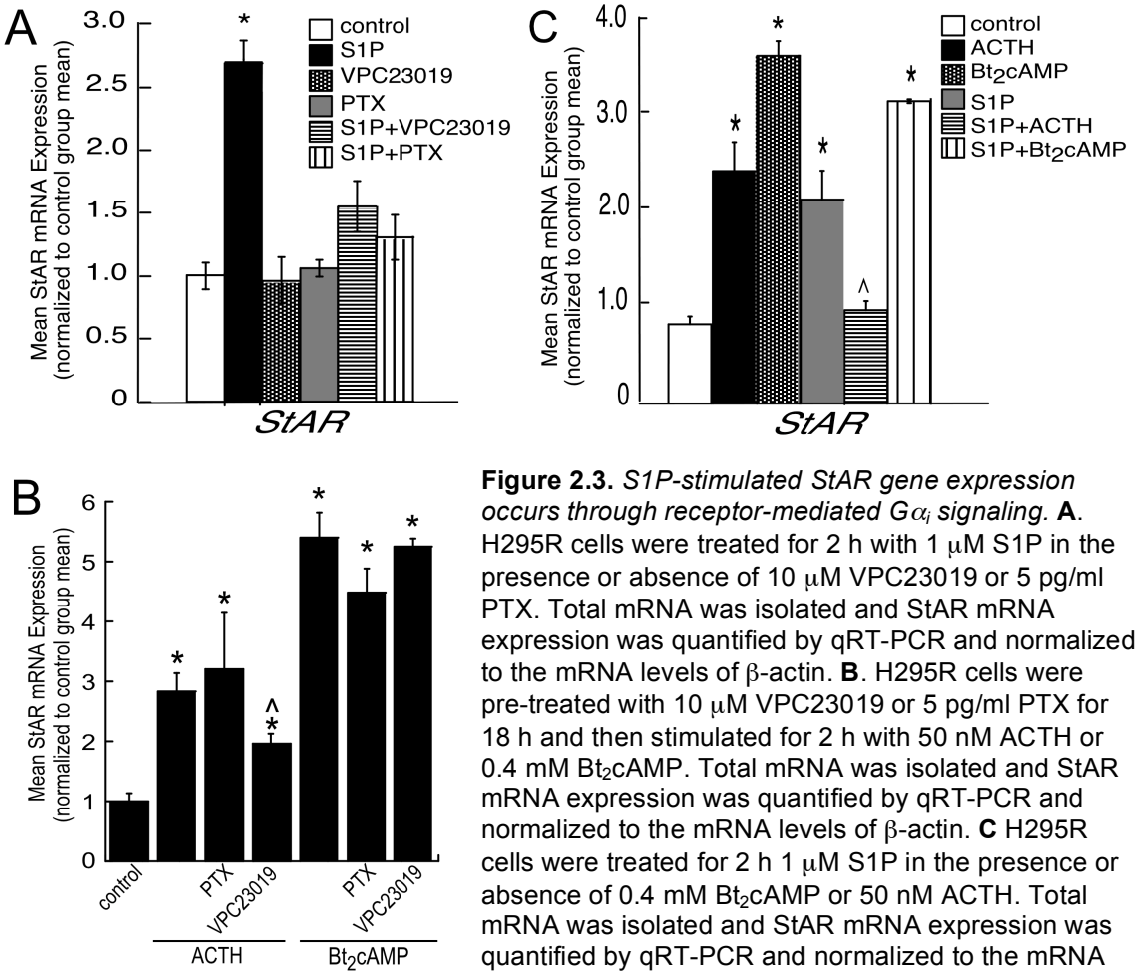



**Figure 2.3.** *S1P-stimulated StAR gene expression occurs through receptor-mediated G$\alpha_i$ signaling.* **A**. H295R cells were treated for 2 h with 1 μM S1P in the presence or absence of 10 μM VPC23019 or 5 pg/ml PTX. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β-actin. **B**. H295R cells were pre-treated with 10 μM VPC23019 or 5 pg/ml PTX for 18 h and then stimulated for 2 h with 50 nM ACTH or 0.4 mM $Bt_2$cAMP. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β-actin. **C** H295R cells were treated for 2 h 1 μM S1P in the presence or absence of 0.4 mM $Bt_2$cAMP or 50 nM ACTH. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β-actin. Data graphed represent the mean ± SEM of 3 experiments, each performed in triplicate. Asterisk (*) and carat (^) denote statistically different ($p < 0.05$) compared to vehicle-treated controls or ACTH-treated, respectively.

effect of the G$\alpha_i$ inhibitor PTX on S1P-dependent StAR gene expression was determined. H295R cells were pretreated with 5 pg/ml PTX, followed by stimulation with 1 μM S1P for 2 h. As shown in Figure 2.3A, PTX prevented S1P-induced expression of StAR. Because multiple S1PR isoforms have been reported to couple to G$\alpha_i$ pathways (309,318,321,338), I next sought to identify the specific S1PR that mediates S1P-induced StAR transcription by pre-treating H295R cells with the $S1PR_1$/$S1PR_3$ antagonist VPC23019 followed by S1P stimulation. VPC23019 abolished S1P-mediated StAR gene expression (Figure 2.3A). Taken together, these data suggest that S1P

acutely activates StAR transcription through activation of $G\alpha_i$-protein coupled receptors $S1PR_1$ and/or $S1PR_3$. Because our laboratory has previously found that ACTH/cAMP signaling promotes the secretion of S1P from H295R cells (295,308), I assessed the effect of PTX and VPC23019 on ACTH/cAMP-stimulated StAR mRNA expression and found that neither inhibitor affected the ability of $Bt_2$cAMP to induce StAR expression (Figure 2.3B). However, VPC23019 significantly reduced ACTH-dependent StAR mRNA levels by 34% (Figure 2.3B). The ability of the $S1PR_{1/3}$ antagonist to partially repress ACTH-stimulated StAR expression is consistent with a second messenger role for S1P in ACTH-dependent steroidogenesis. To further define the relationship between S1P-dependent StAR transcription and ACTH signaling, the effect of S1P on ACTH- and $Bt_2$cAMP-induced on StAR mRNA expression was examined. Neither $Bt_2$cAMP nor ACTH had an additive effect on the S1P response (Figure 2.3C). Unexpectedly, ACTH-stimulated StAR transcription was suppressed in the presence of S1P (Figure 2.3C).

### *2.3.4. S1P-dependent StAR transcription requires PLC activation*

Signaling through $G\alpha_i$ affects multiple downstream effectors including adenylyl cyclase, the small G protein Rac, Src, PI3K/Akt, MAPK, and PLC. The inhibitory effect of PTX and VPC23019 on S1P-mediated StAR mRNA expression suggested that the stimulatory effect of S1P on gene expression occur through the interaction of S1P with $S1PR_{1/3}$. Therefore, in order to identify effectors in the signaling pathway downstream of $G\alpha_i$, the effect of PLC inhibition on S1P-stimulated StAR transcription was determined. H295R cells were pre-treated with 10 μM U73122 followed by treatment with 1 μM S1P for 2 h. PLC inhibition completely abolished S1P-induced StAR mRNA expression, whereas the inactive PLC analog U73343 had no significant effect on the ability of S1P to increase StAR mRNA expression (Figure 2.4A).

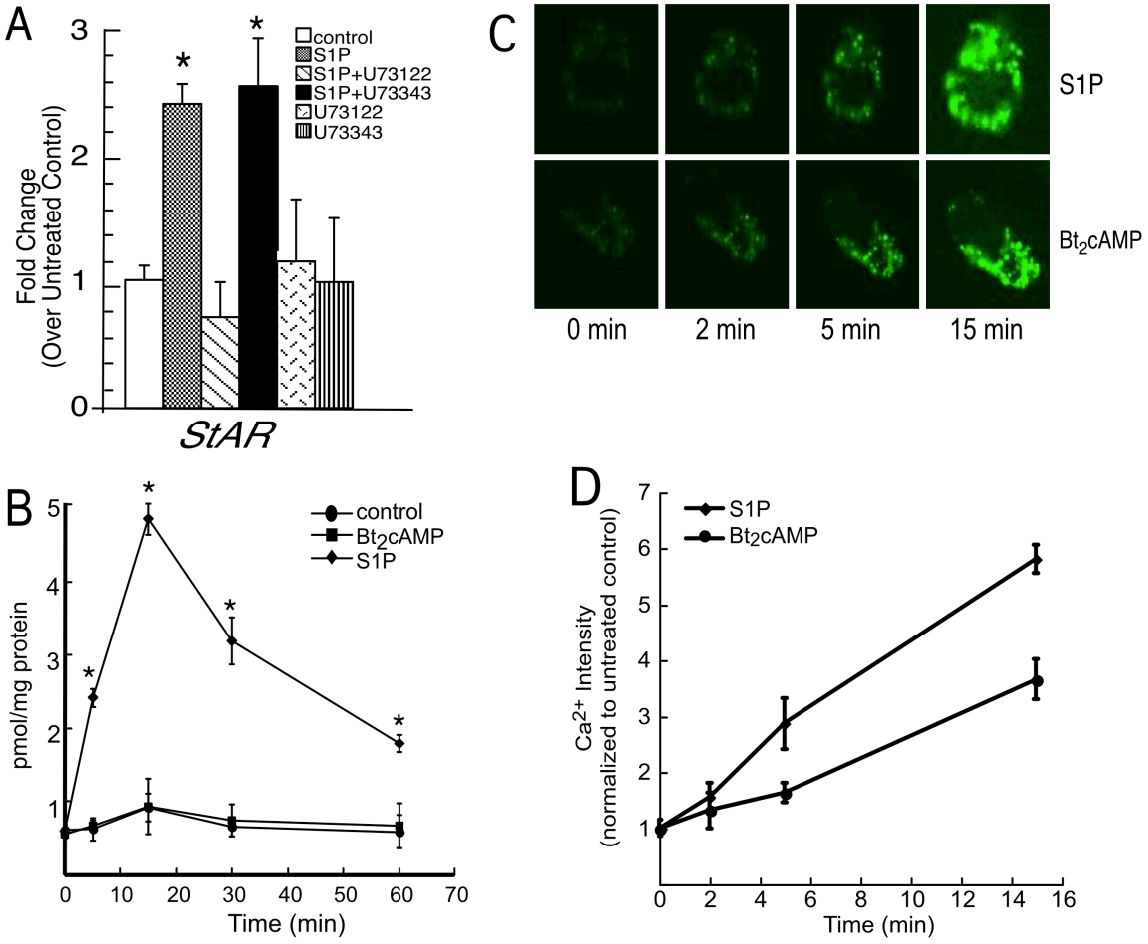



**Figure 2.4.** *S1P-mediated StAR transcription is dependent on PLC activation*. **A.** H295R cells were pretreated with U73122 or U73343 (10 μM) for 1 h and then stimulated for 2 h with 1 μM S1P. Total RNA was isolated and StAR mRNA expression assessed by qRT-PCR and normalized to the mRNA levels of β-actin. Data are graphed as percent of control group mean and represent the mean ± SEM of 3 experiments, each performed in triplicate. Asterisks (*) denote statistically different from the control group ($p < 0.05$). **B.** H295R cells were treated with 1 μM S1P or 0.4 mM $Bt_2cAMP$ for time periods ranging from 5 to 60 min. $IP_3$ content was determined by radioreceptor assay as described in Section 2.2. Data graphed are expressed as pmol of $IP_3$ per milligram of protein and represent the mean ± SEM of 2 experiments, each performed in triplicate. Asterisks (*) denote $p < 0.05$ of S1P-treated cells versus vehicle-treated controls. **C.** Representative immunofluorescence live cell images of cytosolic $Ca^{2+}$ in H295R cells treated for 0 to 15 min with S1P (top panel) or $Bt_2cAMP$ (bottom panel). Cytosolic $Ca^{2+}$ was visualized using Fluo 3/AM (green). **D.** Quantification of cytosolic $Ca^{2+}$ in H295R cells treated for 5 to 15 min with $Bt_2cAMP$ or S1P using the Volocity software. Data are graphed as mean intensity over area and represent the mean ± SEM of 4 experiments, each performed in triplicate.

### *2.3.5. S1P stimulates $IP_3$ release and increases cytoplasmic $Ca^{2+}$*

Based on the findings described above demonstrating that S1P-stimulated StAR gene transcription is dependent on PLC activity, the amount of $IP_3$ release in response to S1P was quantified. H295R cells were treated with 1 μM S1P for 5 to 60 min and $IP_3$ intracellular levels were determined as described in Section 2.2. As shown in Figure 2.4B, S1P significantly increased intracellular $IP_3$ by 2.5- and 4.8-fold after 5 and 15 min, respectively, compared to untreated controls. $IP_3$ levels in S1P-treated cells gradually decreased after 15 min of stimulation, although they remained significantly higher than untreated controls. Notably, $Bt_2$cAMP had no significant effect on $IP_3$ release (Figure 2.4B), suggesting that the mechanism by which S1P induces StAR expression exhibits distinct elements when compared to $Bt_2$cAMP. The increase in $IP_3$ was concomitant with a 2.9- and 5.8-fold increase in cytosolic $Ca^{2+}$ after 5 and 15 min of S1P stimulation, respectively (Figure 2.4D). $Bt_2$cAMP also stimulated an increase in cytosolic $Ca^{2+}$, however, the magnitude of the response was lower than the increase elicited by S1P.

### *2.3.6. Induction of StAR mRNA expression requires CamKII and ERK1/2 activation*

The increases in $IP_3$ and $Ca^{2+}$ led me to postulate that S1P-stimulated StAR expression requires CamKII. To test this hypothesis, H295R cells were treated for 2 h with 1 μM S1P in the presence or absence of the CamKII inhibitor KN-93 (10 μM) and StAR mRNA transcript levels were quantified by qRT-PCR. As shown in Figure 2.5A, inhibiting CamKII activity attenuated S1P-induced StAR mRNA expression. Consistent with this finding, S1P stimulation activated CamKII after 30 min, and pre-treatment with VPC23019 and PTX abrogated S1P-stimulated CamKII phosphorylation (Figure 2.5B).

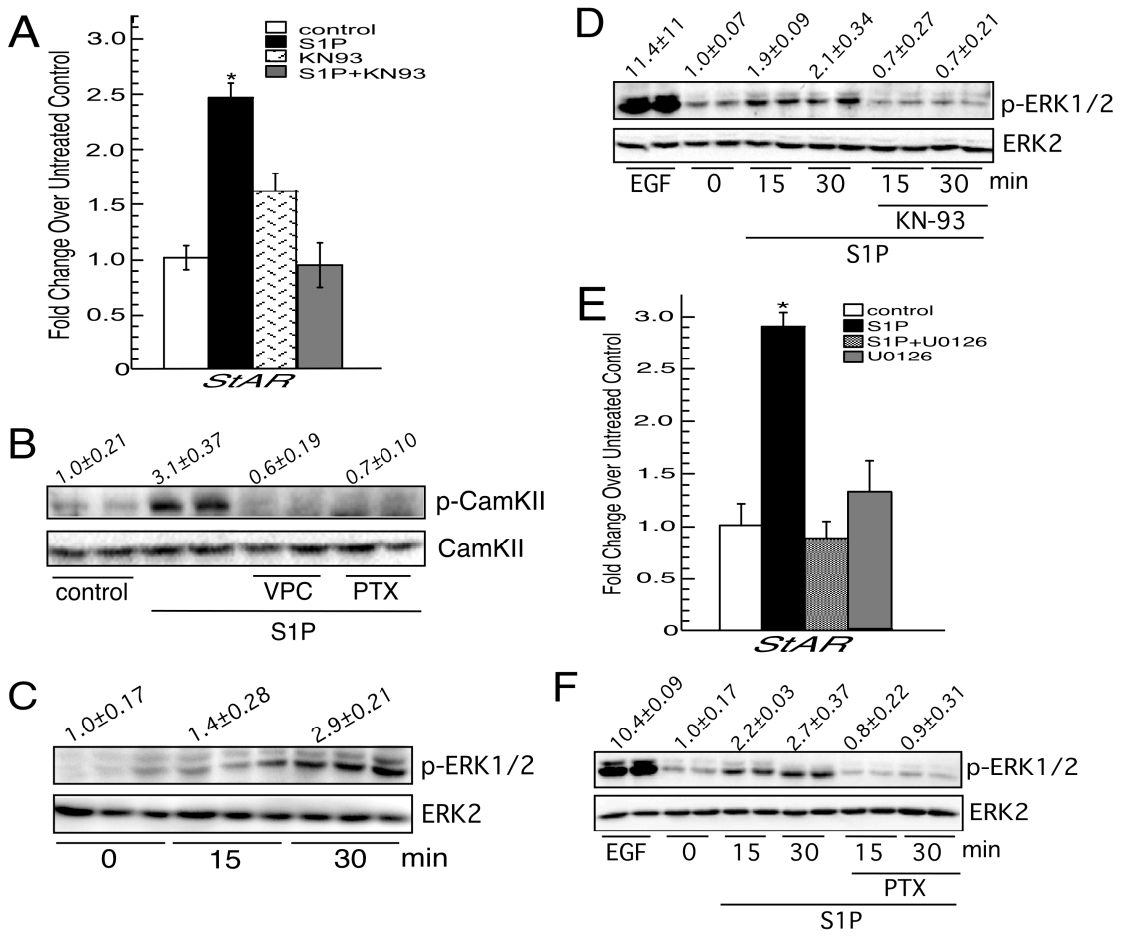



**Figure 2.5.** *S1P-stimulated StAR transcription requires CamKII and ERK1/2 activation.* **A.** H295R cells were pretreated with 10 μM KN-93 and then stimulated with 1 μM S1P for 2 h. Cells were harvested and total RNA isolated for analysis of StAR mRNA expression by qRT-PCR and normalized to the mRNA levels of β-actin. Data graphed are expressed as percent of control group mean and represent the mean ± SEM of 3 experiments, each performed in triplicate. Asterisk (*) denotes $p < 0.05$ for S1P-stimulated mRNA expression versus vehicle-treated control. **B.** H295R cells were pre-treated with 10 μM VPC23019 or 5 ng/mL PTX and then treated with 1 μM S1P for 30 min. Cell lysates were harvested and separated by SDS-PAGE. CamKII activity was assessed in cell lysates by western blotting using an antibody against phospho-$Thr^{286}$-CamKII (top panel) or CamKII (bottom panel). **C**. H295R cells were serum-starved for 30 h, treated for 15 or 30 min with 1 μM S1P, and cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-phospho-ERK1/2 (top panel) and ERK2 (bottom panel) antibodies. **D.** H295R cells were serum-starved for 30 h, pre-treated with 10 μM KN-93 and then treated for 15 or 30 min with 1 μM S1P. Some cells were treated with 25 ng/ml EGF for 10 min. ERK activity was assessed in cell lysates by western blotting using an antibody against phospho-ERK1/2 (top panel) or ERK2 (bottom panel). **E.** H295R cells were treated for 2 h with 1 μM S1P in the presence or absence of 10 μM U0126. Total RNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β-actin. Data graphed represent the mean ± SEM of 3 separate experiments, each performed in triplicate. Asterisk (*) denotes statistically significant difference ($p < 0.05$) compared to vehicle-treated control. **F.** H295R cells were serum-starved for 30 h, pre-treated with 5 pg/ml PTX and then treated for 15 or 30 min with 1 μM S1P. Some cells were treated with 25 ng/ml EGF for 10 min (positive control). Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using antibodies against phospho-ERK1/2 (top panel) or ERK2 (bottom panel).

Because CamKs can crosstalk with many intracellular pathways, including MAPK signaling (339-342), the effect of S1P on the activation of ERK1/2 was investigated. As shown in Figure 2.5C, S1P activates ERK1/2 after 30 min of stimulation. To determine if ERK1/2 activation is upstream of CamKII, the phosphorylation status of ERK1/2 was quantified by western blotting from H295R cells pre-treated with 10 μM KN-93 and stimulated with 1 μM S1P for 15 or 30 min. As shown in Figure 2.5D, pre-treatment with KN-93 prevented ERK1/2 phosphorylation in S1P-treated cells. Additionally, pre-treatment of H295R cells with the MEK1 inhibitor U0126 completely ablated S1P-induced StAR gene expression (Figure 2.5E). Taken together, my results indicate that S1P-dependent StAR transcription requires the sequential activities of CamKII and ERK1/2.

### *2.3.7. S1P-mediated ERK1/2 activation is dependent on $G\alpha_i$ signaling*

My data show that ERK1/2 activation is required for S1P-dependent StAR gene transcription and that CamKII is upstream of ERK1/2. Moreover, $G\alpha_i$ and PLC are also required for the S1P response. Therefore, in order to determine if ERK1/2 phosphorylation occurs through activation of $G\alpha_i$ signaling, western blotting analysis of H295R cells pre-treated with 5 pg/ml PTX and stimulated with 1 μM S1P for 15 or 30 min was carried out. As shown in Figure 2.5F, suppression of $G_i$ signaling by PTX prevents S1P-induced ERK1/2 phosphorylation.

### *2.3.8. S1P acutely increases StAR protein expression and HSL phosphorylation*

To determine if the increase in StAR transcription resulted in an increase in protein expression, StAR protein levels in S1P-treated H295R cells were quantified by western blotting. As shown in Figure 2.6A and 2.6B, S1P significantly increased StAR

protein expression by 1.9- and 2.5-fold after 3 and 6 h of stimulation, respectively. Significantly, although S1P had no effect on HSL mRNA (Figure 2.2B) or protein expression (data not shown), densitometric analysis of western blots revealed that S1P stimulated the phosphorylation of HSL at Ser$^{563}$, a PKA target site that stimulates HSL activity (343-346), by 2.4- and 2.8- fold after 15 and 30 min, respectively (Figure 2.6C and 2.6D).

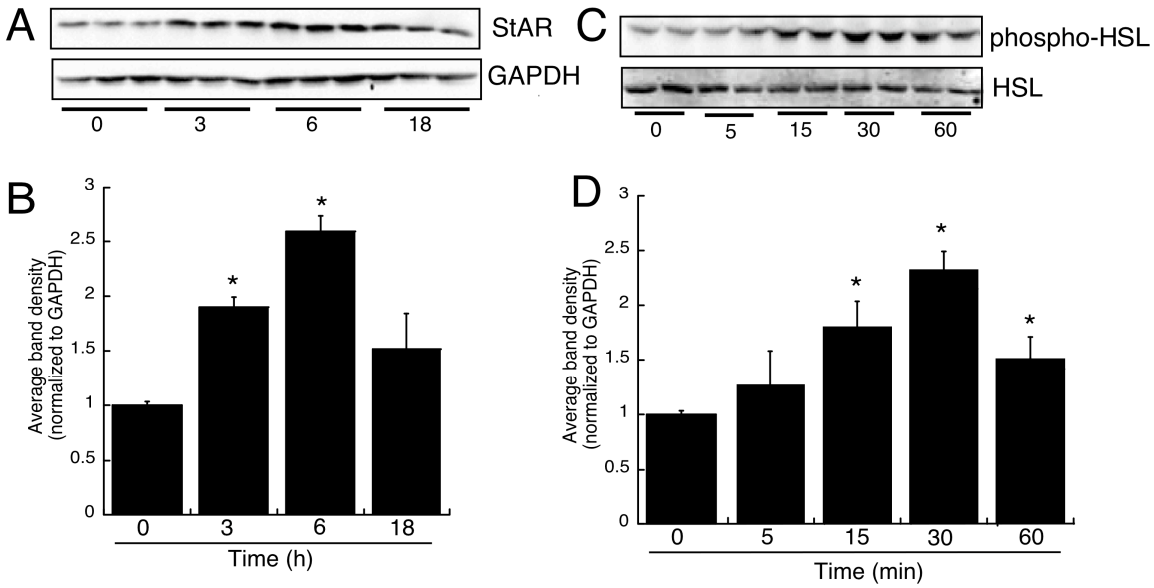



**Figure 2.6.** *S1P increases StAR protein expression and HSL phosphorylation.* **A**. H295R cells were treated with 1 μM S1P for 0 to 18 h and cell lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-StAR and anti-GAPDH antibodies. **B**. Densitometric analysis of western blots of StAR protein expression, normalized to GAPDH protein content in cells treated for 0 to 18 h with 1 μM S1P. Data graphed represent the mean ± SEM of 2 separate experiments, each done in triplicate. Asterisks (*) denote statistically significant difference ($p < 0.05$) versus untreated controls. **C**. H295R cells were treated with 1 μM S1P for 15 to 60 min and cell lysates were harvested and separated by SDS-PAGE followed by western blot analysis using antibodies against phospho-Ser$^{563}$-HSL (top panel) and anti-HSL (bottom panel). **D**. Densitometric analysis of western blots of phospho-HSL protein expression, normalized to HSL protein content in cells treated for 0 to 18 h with 1 μM S1P. Data graphed represent the mean ± SEM of 2 separate experiments, each done in triplicate. Asterisks (*) denote statistically significant difference ($p < 0.05$) versus untreated controls.

## 2.4. Discussion

ACTH promotes glucocorticoid production by coordinating a series of spatially and temporally distinct cellular processes. In what is classically known as the acute phase of steroidogenesis, ACTH-stimulated PKA activation leads to an increase in the hydrolysis of cholesterol esters and the subsequent trafficking of this substrate to the inner mitochondrial membrane, the site of the first enzymatic reaction in the steroid hormone biosynthetic pathway. During the acute phase, ACTH signaling rapidly induces the expression of StAR (278,347) and the activation of HSL (348). Based on previous studies from our laboratory showing ACTH-stimulated sphingolipid metabolism and S1P secretion (308), I sought to define the role of S1P in the acute phase of steroidogenesis. This Chapter describes that S1P induces the transcription of multiple acute steroidogenic genes, stimulates the phosphorylation of HSL at $Ser^{563}$, and increases cortisol production. Further, I show that S1P-stimulated StAR gene expression occurs through an $S1PR_1$/$SIPR_3$-mediated $G\alpha_i$ signaling pathway that involves PLC, CamKII, and ERK1/2 activation.

Although ACTH is the main regulator of cortisol biosynthesis, multiple additional signaling molecules can modulate steroidogenic gene transcription, including growth factors, prostaglandins, and phospholipids (349-352). S1P regulates aldosterone secretion in zona glomerulosa adrenocortical cells (290,307) and stimulates cortisol secretion in zona fasciculata bovine adrenal cells (306). I show that S1P is a novel modulator of acute cortisol biosynthesis in human adrenocortical cells. Although the effect of S1P and $Bt_2$cAMP were similar at earlier time points, the magnitude of the response elicited by $Bt_2$cAMP was significantly greater at later time points (Figure 2.1A). Notably, S1P only increased DHEA secretion transiently at the 6 and 12 h time points (Figure 2.1B). Because the biosynthesis of cortisol requires the movement of intermediate metabolites between the ER and mitochondria and our group has

previously shown that ACTH/cAMP-mediated mitochondrial movement plays a pivotal role in glucocorticoid biosynthesis (353), it is tempting to speculate that S1P is stimulating cortisol production by facilitating substrate delivery to mitochondria. Indeed, our laboratory has found that S1P increases the rate of mitochondrial trafficking (354).

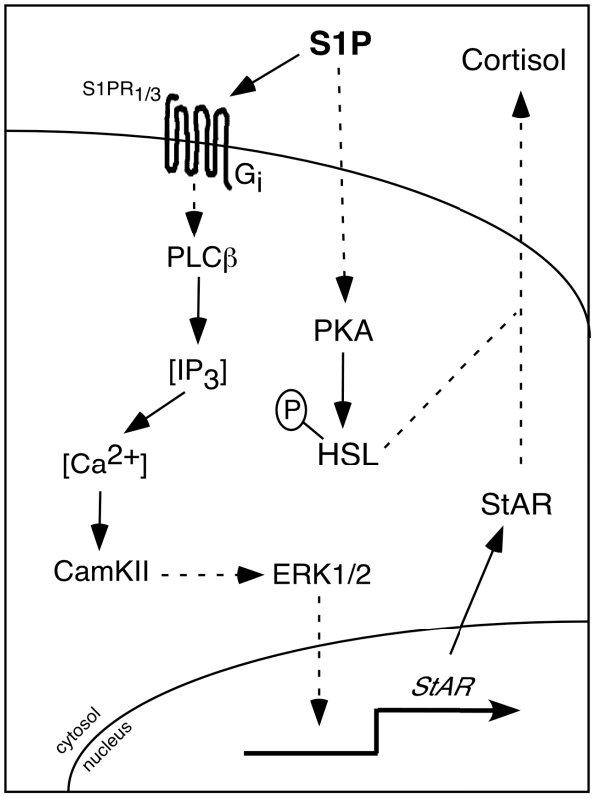



**Figure 2.7**. *Proposed model for S1P-mediated cortisol biosynthesis in H295R cells.* S1P binds to $S1PR_1$ and/or $S1PR_3$ and activates $G\alpha_i$. $G\alpha_i$ couples to PLC, thereby increasing intracellular $IP_3$ and subsequently cytosolic $Ca^{2+}$. Intracellular $Ca^{2+}$ accumulation activates CamKII, which in turn mediates the phosphorylation of ERK1/2. Acute activation of the S1P signaling pathway culminates in an induction of StAR gene expression and an increase in cortisol secretion. S1P also stimulates PKA-mediated phosphorylation of HSL at $Ser^{563}$, which increases cholesterol ester hydrolysis and substrate availability for cortisol production.

During the acute phase of steroidogenesis, ACTH-dependent cholesterol mobilization is mirrored by an increase in the transcription of genes essential for its uptake, transport, and de-esterification, including StAR and TSPO (355). My data show that S1P rapidly increases the transcription of TSPO, SR-BI, LDLR, and StAR (Figure 2.2). Significantly, although S1P had no effect on HSL mRNA expression (Figure 2.2B), phosphorylation of HSL at $Ser^{563}$ was acutely stimulated S1P (Figure 2.6C). As discussed earlier, the activity of HSL is regulated by phosphorylation at multiple residues

(356-359). However, the phosphorylation of HSL at $Ser^{563}$ by PKA (356) triggers HSL translocation to the surface of lipids droplets where it hydrolyzes cholesteryl esters (360-361). Moreover, HSL has been found to interact with StAR (362). Therefore, my data identify multiple roles for S1P in facilitating increased substrate availability and delivery for cortisol biosynthesis (Figure 2.7).

Most of the physiological effects elicited by S1P are the result of binding to tissue-specific S1P receptors (311,363). In this manner, S1P has been shown to activate PLC, PI3K/Akt, and MAPK signaling as well as $Ca^{2+}$ mobilization (290,300,307,320-321,364-365). My results demonstrate that S1P-dependent StAR gene expression occurs through a PTX-sensitive $G\alpha_i$-coupled receptor (Figure 2.3A). Moreover, because the $S1PR_1$/$SIPR_3$ antagonist VPC23019 prevented S1P-induced gene expression, it is likely that S1P signals through one or both of these receptor isoforms. Notably, both isoforms are expressed in the H295R cell line (308) and can couple to $G\alpha_i$ (307,318-319,366). Furthermore, S1P and $Bt_2$cAMP did not have an additive effect on StAR mRNA expression (Figure 2.3C), suggesting that these stimuli activate a common downstream effector. Conversely, S1P abrogated ACTH-stimulated StAR transcription (Figure 2.3C). Because my data suggests that S1P signals through $G\alpha_i$ to induce StAR mRNA expression (Figure 2.3A) and ACTH activates a $G\alpha_s$-cAMP-PKA signaling pathway (367), it is possible that S1P-mediated $G\alpha_i$ activation downregulates ACTH-dependent $G\alpha_s$ signaling by inhibiting adenylyl cyclase (368). $G\alpha_i$-mediated downregulation of cAMP production by S1P has been previously reported (369-372).

Many reports support the activation of $Ca^{2+}$ mobilization by S1P through GPCR-mediated PLC activation (364,373-375). Consistent with these findings, I show that the transcriptional response evoked by S1P requires PLC activation and results in the accumulation of cytoplasmic $IP_3$ and $Ca^{2+}$ (Figure 2.4). It should be noted that S1P has

been shown to increase cytosolic $Ca^{2+}$ by stimulating release from intracellular stores and by activating $Ca^{2+}$ influx (376-378). Although my studies do not distinguish between these two sources, the increase in $IP_3$ levels elicited by S1P (Figure 2.4B) suggest that elevated cytosolic $Ca^{2+}$ in response to S1P stimulation stems, at least in part, from intracellular stores. Notably, even though $Bt_2$cAMP also elevated cytosolic $Ca^{2+}$ levels (Figure 2.4D), it did so independently of cellular $IP_3$ levels (Figure 2.4B), which supports the notion that the molecular mechanism of S1P-mediated glucocorticoid production displays unique components when compared to ACTH/cAMP signaling.

In order to further characterize the signaling molecules involved in the S1P transcriptional response, I show that downstream of PLC, CamKII and ERK1/2 activation are required events for S1P-dependent StAR gene transcription (Figure 2.5). CamKII is a well-established $Ca^{2+}$-activated kinase, thus its activation by increased cytoplasmic $Ca^{2+}$ is plausible. In addition, S1P has been shown to activate this enzyme in vascular smooth muscle cells (377). Consistent with previous reports on the crosstalk between CamK and the MAPK/ERK pathway (339,341-342,379) and the activation of MAPK signaling by S1P (307,380-381), I show that S1P rapidly activated ERK1/2 (Figure 2.5B) in a CamKII-dependent manner (Figure 2.5C). Further, ERK1/2 activation was required for S1P-dependent StAR mRNA expression (Figure 2.5D). Importantly, MAPK/ERK activation in response to $S1PR_1$ and $S1PR_3$ coupling to $G\alpha_i$ has been reported (307,318,366,382-383), further supporting my findings.

ERK1/2-mediated StAR gene expression is well established (384-386) and both PKA and PKC signaling can activate ERK1/2 (386) and StAR transcription (281,386-388). My data identify S1P as a novel regulator of StAR gene expression via the activation of the MAPK/ERK pathway. Regulation of StAR gene transcription involves multiple transcription factors including the bZIP family of transcription factors [CREB/CRE modulator (CREM)/activating transcription factor (ATF)], which binds to

conserved 5'-CRE half-sites (389-390). In addition, increased $Ca^{2+}$ and subsequent activation of CamK leads to CREB phosphorylation in a MAPK-dependent manner (391). Further, S1P was shown to partially activate CREB in this manner (392). Thus, it is tempting to speculate that S1P-induced StAR transcription is mediated, at least in part, through ERK1/2-dependent CREB phosphorylation. Moreover, because ERK1/2 regulate StAR function by phosphorylation at $Ser^{232}$ (393), it is possible that S1P stimulation triggers StAR phosphorylation in addition to inducing its gene expression. Future studies are needed to test these hypotheses as well as uncover other regulatory proteins involved in S1P-dependent acute steroidogenic gene expression.

Our laboratory (308) and others (290,306-307) have reported that S1P mediates steroid hormone biosynthesis by activating various intracellular cascades. Rabano *et al. (175)* demonstrated that S1P-mediated cortisol secretion in bovine adrenocortical cells is dependent on PKC and intracellular $Ca^{2+}$ in a PTX-sensitive manner. My results support a similar mechanism of action for S1P in human H295R cells, although I demonstrate that PLC activation is also required. Importantly, S1P induces the phosphorylation of HSL at $Ser^{563}$, providing evidence that S1P regulates steroidogenesis by acting via multiple pathways. Given that ACTH/cAMP promotes the secretion of S1P from H295R cells (308), it is plausible that adrenocortical cells might utilize an ACTH/S1P feed-forward mechanism to facilitate rapid steroidogenic output and fine-tune sustained hormone production. In summary, I demonstrate that S1P acts at multiple levels to promote cortisol production.

## CHAPTER 3:

*The cAMP responsive element binding protein (CREB) regulates the expression of acid ceramidase in H295R human adrenocortical cells*

### 3.1. Introduction

Bioactive sphingolipids such as Cer, SPH, and S1P have been implicated in steroidogenesis. Cer suppresses progesterone secretion in ovarian granulosa cells (207) and rat luteal cells (212), decreases testosterone production in rat gonadal Leydig cells (205,210), modulates P450c17α enzymatic activity in rat Leydig cells (205), and inhibits hCG-induced P450 aromatase (CYP19A1) activity and estradiol production in ovarian granulosa cells (206,394). Our laboratory has shown that in H295R cells, suppression of ASAH1 expression leads to increased transcription of the CYP17A1 gene, indicating a role for this ceramidase in adrenocortical steroidogenesis (166). Furthermore, we have also demonstrated that SPH inhibits CYP17A1 transcription and cortisol biosynthesis by acting as an antagonistic ligand for SF-1 (26-27). SPH can be rapidly phosphorylated by SPHKs to form S1P, which mediates $Bt_2$cAMP-stimulated CYP17A1 transcription in H295R cells (395), increases cortisol secretion in bovine fasciculata cells (175), and stimulates aldosterone secretion in bovine glomerulosa cells (176,396). In addition to studies demonstrating that sphingolipids regulate steroidogenesis, trophic factors that activate steroid hormone biosynthesis (e.g. ACTH) have been found to modulate sphingolipid metabolism. In H295R cells, ACTH stimulates sphingolipid metabolism by rapidly promoting the catabolism of SM and Cer. ACTH acutely activates SPHK activity, thus increasing S1P concentrations (166,395,397). Collectively, these data highlight the intimate, reciprocal relationship between sphingolipid metabolism and steroid hormone biosynthesis.

CREB proteins are leucine zipper-containing transcription factors that regulate the expression of several genes by binding to CRE sequences at target promoters (398-

399). In response to cAMP signaling, PKA phosphorylates CREB at $Ser^{133}$, a post-translational modification that is essential for its transcriptional activity (398,400-401). CREB binds to the promoter of target genes and facilitates the recruitment of coactivators, including CREB binding protein (CBP/p300) (402-404) and transducer of regulated CREB-binding proteins (TORCs) (405-406) by a mechanism that is either dependent (e.g. CBP/p300) or independent (e.g. TORCs) of $Ser^{133}$ phosphorylation. In addition to activating target gene transcription, CREB can also mediate transcriptional repression by partnering to repressor proteins. For instance, Kibler and Jeang (407) reported that a CREB/ATF-dependent cyclin A repression occurs through a protein-protein interaction with the human T cell leukemia virus type 1 Tax protein. Furthermore, the transcription factor YY1 represses C-FOS transcription by forming a complex with CREB/ATF on the DNA (408).

Based on previous data from our laboratory identifying SPH as an antagonist for SF-1 and the effect of ACTH-stimulated sphingolipid metabolism on steroidogenic gene transcription and hormone output, I sought to determine the role of ACTH/cAMP signaling in regulating the expression of the ASAH1 gene in H295R cells. I identify ASAH1 as a CREB-responsive gene and show that CREB is essential for $Bt_2$cAMP-stimulated ASAH1 transcription. Moreover, CREB enrichment at multiple sites on the ASAH1 promoter facilitates the recruitment of CBP and p300 as well as histone H3 lysine 4 (H3K4) trimethylation. Finally, I demonstrate that $Bt_2$cAMP-mediated ASAH1 transcription lead to a significant increase in protein expression and enzymatic activity, thus supporting a role for ASAH1 as an important enzyme in the regulation of cortisol biosynthesis.

## 3.2. Materials and Methods

### *3.2.1. Cell culture*

H295R adrenocortical cells were cultured as described in Chapter Two.

### *3.2.2. Real time RT-PCR (qRT-PCR)*

Cells were sub-cultured into 12-well plates and 48 h later treated with 0.4 mM $Bt_2$cAMP for 1-24 h. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Fisher Scientific Inc, Waltham, MA) and the primers listed in Table 3.1. ASAH expression was normalized to β-actin content and calculated using the delta-delta cycle threshold (ΔΔCT) method.

### *3.2.3. Cloning of the human ASAH1 promoter and site-directed mutagenesis*

The human ASAH1 promoter was cloned using LA *Taq* DNA polymerase (Takara, Madison, WI), 500 ng of human genomic DNA (Promega, Madison, WI) and 300 nM of the primers listed in Table 3.1. PCR fragments were then cloned into the pGL3 (Promega) reporter gene plasmid at the *Mlu I* (5') and *Bgl II* (3') sites. The pGL3-ASAH1 constructs were confirmed by sequencing prior to use in transient transfection experiments. *In silico* analysis using the MatInspector software (Genomatix Software, Ann Arbor, MI) was used to identify putative consensus binding sites for CREB on the ASAH1 promoter.

### *3.2.4. Transient transfection and reporter gene analysis*

H295R cells were sub-cultured into 24-well plates and transfected with 150 ng of pGL3-ASAH1, 1.5 ng pRL-TK (Promega), and/or 5 ng of pCMV-CREB (BD Biosciences, Franklin Lakes, NJ) or 5 ng of pCMV-K-CREB (BD Biosciences) using GeneJuice (Novagen, Madison, WI). Twenty-four hours after transfection, the cells were treated with

0.4 mM $Bt_2$cAMP for 16 h and the transcriptional activity of the ASAH1 reporter gene determined using a dual luciferase assay kit (Promega). *Firefly* (pLG3-ASAH1) luciferase activity was normalized to *Renilla* luciferase activity (pRL-TK) and expressed as fold change over the mean of the untreated control group.

### *3.2.5. Electrophoresis mobility shift assay (EMSA)*

Nuclear extracts were isolated from H295R cells that were incubated with 0.4 mM $Bt_2$cAMP for 1 h using the NE-PER kit (Pierce, Rockford, IL). Double-stranded oligonucleotides (Table 3.1) were labeled with [α-$^{32}$P] dCTP (MP Biomedicals, Solon, OH) using DNA polymerase I, Klenow fragment (Stratagene, La Jolla, CA). For EMSA reactions, 5 μg of nuclear protein, 0.5 μg poly(dI•dC), 50 μg BSA, and 10,000 CPM of $^{32}$P-labeled probe were mixed in 25 μL binding buffer [20 mM HEPES (pH 7.9), 80 mM KCl, 5 mM $MgCl_2$, 2% Ficoll, 5% glycerol, 0.1 mM EDTA, and 0.2 mM dithiothreitol] at room temperature. For supershift assays, 1 μg of anti-CREB antibody (sc-240X, Santa Cruz) was incubated with nuclear extracts, BSA, and poly(dI•dC) for 20 min at room temperature. Labeled probe was added and the reactions were incubated for an additional 15 min at room temperature. In some reactions, 500 ng of recombinant CREB protein (Biomol International, Plymouth Meeting, PA) was used instead of nuclear extracts. DNA-protein complexes were resolved on 5% polyacrylamide/0.5% Ficoll/0.5X TBE gels and the dried gels exposed to a phosphoimager screen. Complexes were visualized by prosphoimager scanning (Phospho/Fluorimager, Fuji Film, Japan).

### *3.2.6. Chromatin immunoprecipitation (ChIP)*

ChIP assays were performed as described in Dammer *et al.* (158). Briefly, H295R cells were sub-cultured into 150-mm dishes and treated with 0.4 mM $Bt_2$cAMP for 30 or 60 min. The purified chromatin solutions were immunoprecipitated using 5 mg

of anti-phospho-Ser$^{133}$-CREB, anti-CBP, anti-p300, and anti-trimethyl H3K4. Real-time PCR was carried out using the primer sets indicated in Table 3.1 and output (immunoprecipitated promoter region) normalized to input DNA. Some reactions were also subjected to agarose (4%) gel electrophoresis.

### *3.2.7. RNA interference (RNAi) and real time RT-PCR (qRT-PCR)*

Cells were sub-cultured into 12-well plates and 24 h later transfected with 75 nM of nonspecific small interfering RNA (siRNA) oligonucleotides or siRNA oligonucleotides directed against CREB (SI00299894, Qiagen, Valencia, CA) using HiPerfect Transfection Reagent (Qiagen). After 48 h, cells were treated with 0.4 $Bt_2$cAMP for 24 h. Total RNA was extracted using TRIzol (Invitrogen) and ASAH1 mRNA expression was quantified using a One-Step SYBR Green RT-PCR Kit (Thermo Scientific) and normalized to β-actin. Western Blotting was performed to confirm reduction of CREB protein expression.

### *3.2.8. Western blotting*

H295R cells were treated with 0.4 mM $Bt_2$cAMP for 24, 48, or 72 h and harvested into RIPA buffer. Cells were then lysed by sonication (one 5 sec burst) followed by incubation on ice for 30 min. Lysates were centrifuged for 10 min at 4°C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (30 μg of protein) were run on 10% SDS-PAGE gels and transferred to PVDF membranes (Thermo Scientific, Rockford, IL). Blots were probed with an anti-ASAH1 (HPA005468, Sigma, St. Louis, MO) and anti-GAPDH (Santa Cruz) antibodies and expression was detected using an ECF western blotting kit (GE Healthcare, Piscataway, NJ) and visualized by scanning blots on a Typhoon Trio Scanner (GE Healthcare). Protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay (Pierce).

### *3.2.9. In vitro acid ceramidase activity assay*

Activity assays were performed as described in Nikolova-Karakashian and Merrill (409). H295R cells were sub-cultured into 100-mm dishes as treated for 24, 48, or 72 h with 0.4 mM $Bt_2$cAMP. After treatment, cells were harvested into lysis buffer (0.2% Triton X-100, 10 mM Tris-Cl, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA, 15 mM NaCl) with protease inhibitors (EMD Chemicals, Gibbstown, NJ) and sonicated 5 times for 2 sec burst. Protein concentrations were determined using the BCA Protein Assay (Pierce). Acid ceramidase activity was assayed by incubating 100 μL of cell lysate (at least 15 μg/μL of proteins) with 2 μL of a 1 mM NBD-12-cer stock in 0.5 M acetate buffer (pH 4.5) for 2 h at 37°C. Reactions were terminated by the addition of 10 μL oleic acid (10 mg/mL), 1mL chloroform:methanol (2:1, v/v), and 1 mL Dole's solution (isopropanol:heptane:2 N $H_2SO_4$, 40:10:1, v/v/v) followed by vortexing and a 10 min incubation at room temperature. Four hundred μL heptane and 600 μL distilled $H_2O$ was added, the mixtures were vortexed for 2 min, and then centrifuged for 10 min at 4,000 rpm. The lower organic phase was dried under a stream of nitrogen and spotted on Silica Gel 60 thin-layer chromatogray (TLC) plates (EMD Chemicals). Plates were developed in chloroform:methanol:25% $NH_4OH$ (90:20:0.5, v/v/v) and visualized by fluorescence scanning on a Typhoon Trio Scanner (GE Healthcare). NBD-dodecanoic acid formation was quantified by densitometry and normalized to the protein content of each sample.

### *3.2.10. Statistical analysis*

One-way ANOVA, Tukey-Kramer multiple comparison, and unpaired student t-tests were performed using GraphPad InStat software (GraphPad Software Inc., San

Diego, CA). Significant differences from a compared value were defined as $p < 0.05$ and denoted by asterisks (*) or carats (^).

**Table 3.1.** Primer and oligonucleotide sequences used for ChIP, RT-PCR, EMSA, and cloning.

| Method | Region | Forward primer | Reverse primer |
|---|---|---|---|
| ***ChIP*** | -1779/-1596 | TTCCCGGGTTCACGCCAT | GCGCGGTGGCTCACGCCTGTAA |
| | -1475/-1364 | CTCCCTTTTCCTCCACTGCATTTGT | ACAATGTGCCAAGCATGTCTCCTGACAC |
| | -1022/-899 | CGGCAGCGTGCTGAGCTTCATCAAAGC | CAGTCGCGCGGGTAGGTGACCGGGTTGG |
| | -325/-214 | ACGGGTGAAGCTCCCGGCCCCACCTA | GAAAAGGGTGGCGTAGAGAAAGAGAGA |
| | -123/+31 | AGTCCCGCCTCCTCCGAGCGTTCCCCCT | GACTAAGGCGACGCAACTCCGGCCCGGC |
| ***RT-PCR*** | β-actin | ACGGCTCCGGCATGTGCAAG | TGACGATGCCGTGCTGCATG |
| | ASAH1 | GCACAAGTTATGAAGGAAGCCAAG | TCCAATGATTCCTTTCTGTCTCG |
| ***EMSA*** | -281/-261 | CGAGGGGGATGGATCACGCCAGCCGC | CGGCGGCTGGCGTGATCCATCCCCCT |
| | -969/-949 | CGCTGCTTGAGACGTCAGAGGCT | CGAGCCTCTGACGTCTCAAGCAG |
| | -1106/-1086 | CGGAGTGTTGAGTTTTGTAAAGAAATAATAC | CGTGTATTATTTCTTTACAAAACTCAACACTC |
| | -1565/-1545 | CGCCTGTCCCTCTTATTTAAAATTGTAACTCTACCACTTCTGATCTCCACAC | CGGTGTGGAGATCAGAAGTGGTAGAGTTACAATTTTAAATAAGAGGGACAGG |
| | -1776/-1756 | CGTTCCCGGGTTCACGCCATTCTCCTGCCTCA | CGTGAGGCAGGAGAATGGCGTGAACCCGGGAA |
| | -1861/-1841 | CGAGTTTCATTTTTATGTGACGGAGTCTCGCACTGGCGCGCA | CGTGCGCGCCAGTGCGAGACTCCGTCACATAAAAATGAAACT |
| ***ASAH1 cloning*** | -2739 | CGAGCTCTTACGCGTTACCATTTTCTATGAACA | CTTAGATCGCAGATCTGGCGGCAGCCAGGAGGAC |
| | -1430 | TCACCGAGAACATACGCCTCAG | CTTAGATCGCAGATCTGGCGGCAGCCAGGAGGAC |
| | -906 | CTTTGAAATCCAACCCGGTCCC | CTTAGATCGCAGATCTGGCGGCAGCCAGGAGGAC |
| | -496 | CGCTTTTCTCAGAGGGCAAAG | CTTAGATCGCAGATCTGGCGGCAGCCAGGAGGAC |
| | -120 | TGGAATGGTGCGGTCCCAGGTC | CTTAGATCGCAGATCTGGCGGCAGCCAGGAGGAC |

## 3.3. Results

### *3.3.1. $Bt_2$cAMP regulates ASAH1 mRNA expression*

As discussed earlier, steroidogenesis in adrenocortical cells is activated by a cAMP-dependent pathway. There are 5 genes that encode ceramidase enzymes (ASAH1, ASAH2, ACER1-3). Therefore, the effect of increased intracellular cAMP on ASAH gene expression in H295R cells was determined by treating with 0.4 mM $Bt_2$cAMP for 24 h. ASAH1 mRNA expression was increased by 2.8-fold after 24 h treatment with $Bt_2$cAMP (Figure 3.1A). Conversely, no increase in expression in response to $Bt_2$cAMP was observed for both ASAH2 and ACER3 (Figure 3.1A), indicating that the cAMP signaling pathway differentially regulates the expression of these genes. Of note, similar results were obtained for mouse Y1 adrenocortical cells (data not shown). Next, I assessed the kinetics of the ASAH1 response to $Bt_2$cAMP by treating H295R cells for 1

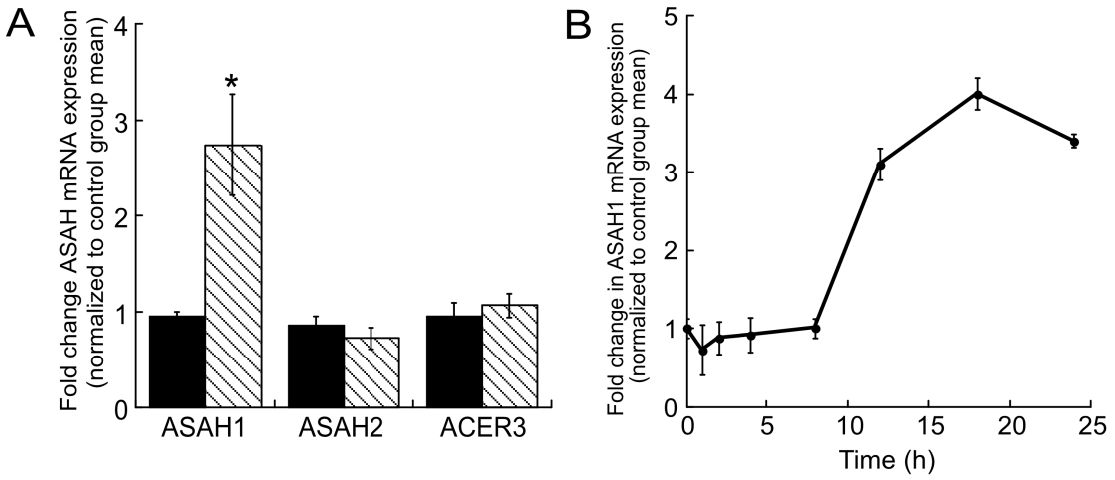



**Figure 3.1.** *$Bt_2$cAMP increases ASAH1 mRNA expression.* **A.** H295R cells were cultured into 12-well plates and treated for 24 h with 0.4 mM $Bt_2$cAMP. Total RNA was isolated for analysis of ASAH1, ASAH2, or ACER3, and β-actin mRNA expression by qRT-PCR. Data is graphed as fold change in ASAH mRNA expression and normalized to the mRNA expression of β-actin. Data graphed represent the mean ± SEM of 3 separate experiments, each performed in triplicate. *; Statistically different from untreated control group, $p < 0.05$. **B.** H285R cells were treated for 1 to 24 h with 0.4 mM $Bt_2$cAMP and ASAH1 and β-actin mRNA expression quantified by qRT-PCR. Data is graphed as fold change in ASAH1 mRNA content and is normalized to the mRNA expression of β-actin. Data graphed represent the mean ± SEM of 3 separate experiments each performed in triplicate*; Statistically different from untreated control group, $p < 0.05$.

to 24 h. These temporal experiments revealed that $Bt_2$cAMP evoked a rapid and transient decrease in ASAH1 mRNA expression within 1 h, followed by a chronic increase in mRNA expression (Figure 3.1B). A maximal 4-fold increase in ASAH1 mRNA expression was observed at the 18 h time point.

### *3.3.2. CREB increases ASAH1 reporter gene activity*

To further define the mechanism by which $Bt_2$cAMP modulates ASAH1 mRNA expression, 2.7 Kb of the human ASAH1 promoter was cloned and the amplified product ligated into the pGL3 vector. H295R cells were transfected with this reporter construct for 24 h followed by exposure to 0.4 mM $Bt_2$cAMP for 16 h. As shown in Figure 3.2, $Bt_2$cAMP treatment significantly increased the transcriptional activity of the 2.7 Kb reporter gene by 1.5-fold. Because I observed a significant increase in ASAH1 transcription in response to $Bt_2$cAMP treatment, I performed *in silico* analysis of the 2.7

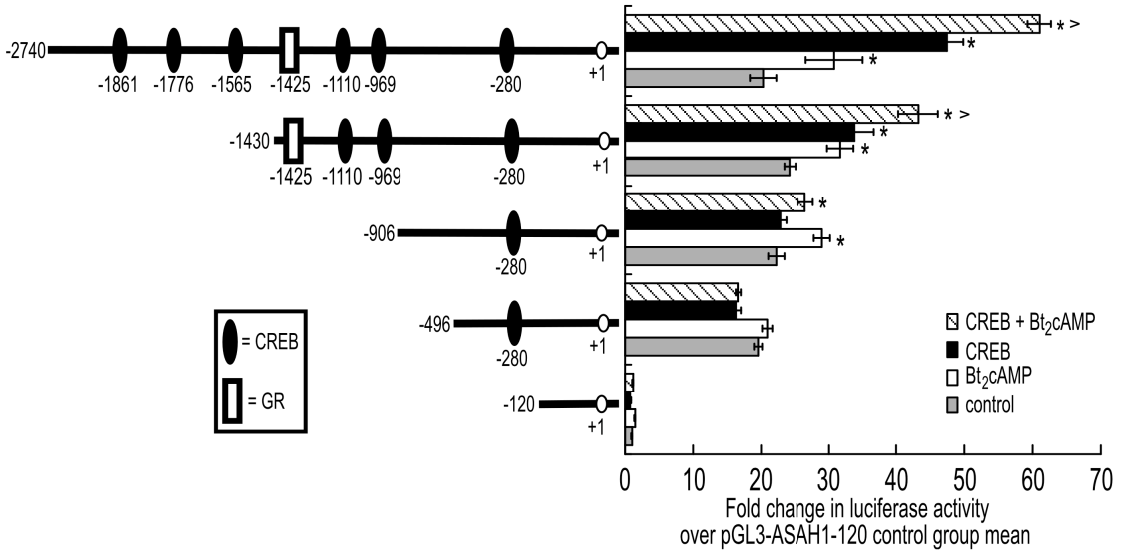



**Figure 3.2.** *Localization of the cAMP- and CREB-responsive region(s) of the ASAH1 promoter.* H295R cells were transfected with reporter gene plasmids (pGL3-ASAH1) containing varying regions of the ASAH1 promoter and a CREB expression plasmid using GeneJuice as described in Section 3.2. Twenty-four hours after transfection, cells were treated with 0.4 mM $Bt_2$cAMP for 16 h and luciferase activity in the cell lysates quantified by luminometry. Statistical difference from untreated control within each transfection group or between control and $Bt_2$cAMP-treated cells transfected with CREB is denoted by * and ^, respectively; $p < 0.05$.

Kb ASAH1 promoter and found 6 putative CREs (Figure 3.2). Overexpression of CREB increased luciferase activity of the 2.7 Kb construct by 2.3 fold when compared to cells transfected with only pGL3-ASAH1(-2740). $Bt_2$cAMP stimulation resulted in a further 30 % increase in reporter gene activity over the CREB-mediated activation in luciferase expression.

To define the cAMP-responsive region(s) of the ASAH1 promoter, deletion constructs were generated and assayed in transfection assays. While deletion of region -2740 to -906 had no significant effect on the ability of $Bt_2$cAMP to stimulate reporter gene activity, removal of 410 base pairs (-496 construct) completely attenuated the $Bt_2$cAMP response (Figure 3.2). Deletion of the region encompassing -2740 to -1430 decreased the stimulatory effect of CREB overexpression by approximately 33 % in both control and $Bt_2$cAMP-treated cells and deletion of the region encompassing -1430 to -906 completed ablated the CREB response. The integral role of CREB was confirmed in reporter gene studies using a dominant-negative CREB (pCMV-K-CREB) (410) which

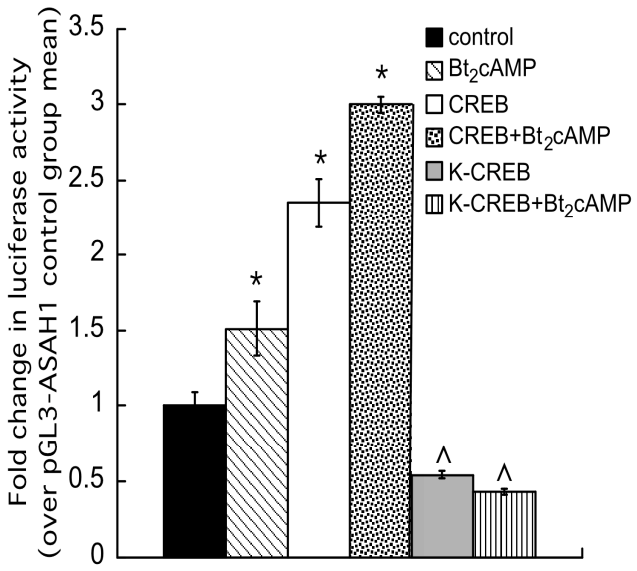



**Figure 3.3.** *Dominant-negative mutant CREB abrogates ASAH1 reporter gene activity.* H295R cells were transfected with pGL3-ASAH1(-2740), wild type (pCMV-CREB) or dominant negative mutant (pCMV-K-CREB) CREB expression plasmids, and pRL-TK. Luciferase activity in lysates isolated from control and $Bt_2$cAMP-treated cells was quantified by luminometry. Data are expressed as the fold change in pGL3-ASAH1(-2740) reporter gene activity over the untreated control group mean and represent the mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) and carats (^) denote a statistically significant difference ($p < 0.05$) from the untreated control group and the untreated CREB-transfected group, respectively.

completely attenuated the stimulatory effects of both CREB and $Bt_2$cAMP (Figure 3.3). Notably, dominant negative CREB also reduced basal luciferase activity by 50 %, suggesting that CREB may play a role in regulating the constitutive expression of ASAH1.

### *3.3.3. CREB is required for $Bt_2$cAMP-stimulated ASAH1 mRNA expression*

In order to investigate if CREB was required for ASAH1 transcription, RNAi was used to suppress CREB translation (Figure 3.4A) and the effect of reduced CREB expression on $Bt_2$cAMP-stimulated ASAH1 transcription was assessed. As shown in Figure 3.4B, H295R cells transfected with CREB siRNA oligonucleotides lose the ability to respond to $Bt_2$cAMP.

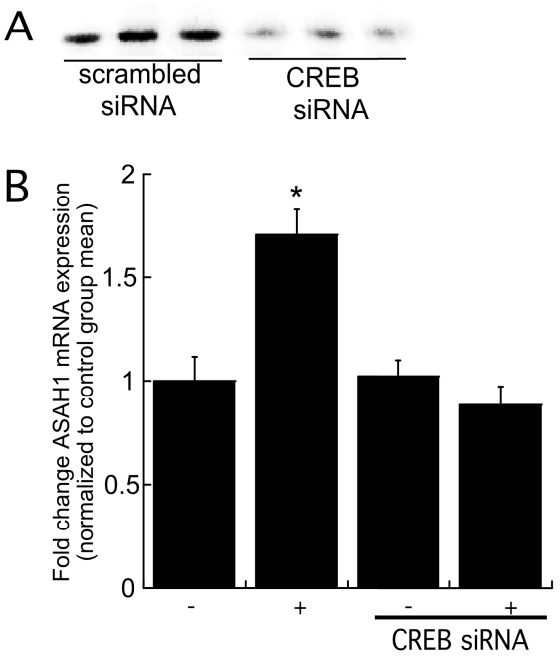



**Figure 3.4.** *Silencing CREB decreases $Bt_2$cAMP-stimulated ASAH1 transcription*. **A.** H295R cells were transfected with 75 nM CREB or scrambled siRNA oligonucleotides for 72 h and total cell lysates were isolated for SDS-PAGE and western blotting. Blots were hybridized to an anti-CREB antibody. **B.** RNA isolated from untreated or $Bt_2$cAMP-stimulated cells that were transfected with CREB siRNA were subjected to qRT-PCR. Data is graphed as fold change in ASAH1 mRNA expression and is normalized to the mRNA expression of β-actin and represent the mean ± SEM of three separate experiments, each performed in triplicate. -, Control; +, $Bt_2$cAMP. (*) indicates a statistically significant difference compared to untreated controls ($p < 0.05$).

### *3.3.4. CREB binds to the ASAH1 promoter in vitro and $Bt_2$cAMP strengthens this binding*

My reporter gene assay with ASAH1 deletion constructs indicated that CREs located between -2750 and -906 confer CREB responsiveness (Figure 3.2). Therefore,

EMSA was carried out to determine which of the putative CREs interacted with CREB. Radiolabeled double-stranded oligonucleotides corresponding to the 6 regions depicted in Figure 3.5A (sequences in Table 3.1) were incubated with either recombinant CREB or nuclear extracts isolated from control or $Bt_2$cAMP-treated cells. As shown in Figure 3.5B, specific DNA-protein complexes were found with 4 of the 6 probes tested (-1861/-1841, -1776/-1756, -969/-949, -281/-261). The putative CREs at -1861/-1841 (panel 1, lane 1) and -1776/-1756 (panel 2, lane 1) exhibited weak interaction with recombinant CREB when compared to the -969/-949 (panel 3, lane 1) probe.

A weak upper and more intense lower band were revealed in reactions containing recombinant CREB and-1861/-1841 or -1776/-1756 (lane 1 in panels 1 and

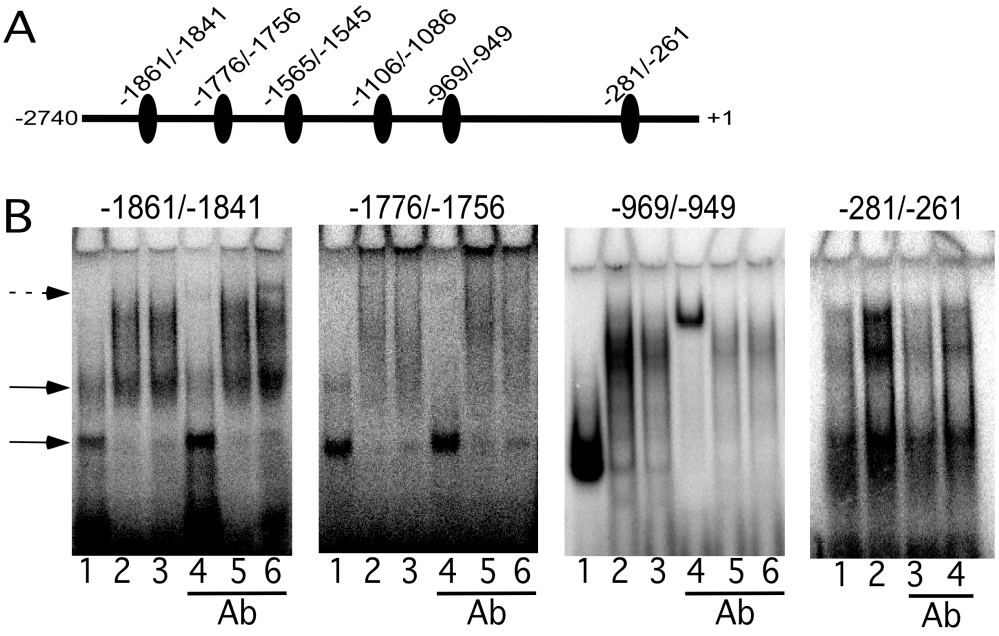



**Figure 3.5.** *CREB binds to the ASAH1 promoter in vitro*. **A.** Depiction of the regions of the ASAH1 promoter corresponding to each oligonucleotide probe. Ovals indicate putative CREB binding sites. **B.** Nuclear extracts isolated from cells treated with 0.4 mM $Bt_2$cAMP for 1 h or recombinant CREB (0.5 mg) were incubated with $^{32}$P radiolabeled oligonucleotides (10,000 cpm) corresponding to the following regions of the ASAH1 promoter: -1861/-1841, -1776/-1756, -969/-949, and -281/-261. Lanes in panels 1 – 3: 1) recombinant CREB, 2) control nuclear extracts, 3) $Bt_2$cAMP-treated nuclear extracts, 4) recombinant CREB + anti-CREB antibody, 5) control nuclear extracts + anti-CREB antibody, 6) $Bt_2$cAMP-treated nuclear extracts + anti-CREB antibody. Panel 4: untreated nuclear extracts (lane 1), $Bt_2$cAMP-treated nuclear extracts (lane 2), untreated nuclear extracts + anti-CREB antibody (lane 3), $Bt_2$cAMP-treated nuclear extracts + anti-CREB antibody (lane 4). Solid arrows indicate CREB binding and dashed arrow denotes shifted bands.

2), suggesting the interaction of monomeric (lower band) and dimeric (upper band) CREB with these regions of the ASAH1 promoter. In contrast, the -969/-949 probe exhibited one major DNA-protein complex when incubated with recombinant CREB (panel 3, lane 1). A supershift was observed when anti-CREB antibody was added to the reaction containing recombinant CREB and the -969/-949 probe (panel 3, lane 4). Further, when nuclear extracts were incubated with the -969/-949 probe in the presence of anti-CREB antibody, a significant reduction in DNA-protein complex formation was observed (panel 3, lanes 5 and 6). Notably, the mobility of DNA-protein complexes formed when the -969/-949 region was incubated with nuclear proteins (panel 3, lanes 3 and 4) was significantly retarded when compared to reactions containing recombinant CREB (panel 3, lane 1). This difference in mobility suggests that CREB may be a component of a multi-protein complex.

A weak supershift was also found when the antibody was added to reactions containing the -1861/-1841 probe and nuclear extracts isolated from $Bt_2$cAMP-treated cells (panel 1, lane 6). $Bt_2$cAMP treatment increased the affinity of nuclear proteins for the -281/-261 region (compare lanes 1 and 2 in panel 4) and the CREB antibody decreased cAMP-stimulated complex formation (compare lanes 2 and 4 in panel 4). No significant binding to -1565/-1545 and -1110/-1086 oligonucleotides was observed (data not shown). Additionally, no changes in the formation of DNA-protein complexes were observed when reactions contained antibodies against ATF-1 or ATF-2 (data not shown).

### *3.3.5. $Bt_2$cAMP promotes the recruitment of CREB and other coregulatory proteins to the ASAH1 promoter*

I next examined the effect of $Bt_2$cAMP stimulation on the recruitment of CREB and coactivator proteins to the endogenous ASAH1 promoter (Figure 3.6A) by

performing chromatin immunoprecipitation (ChIP) assays using chromatin isolated from H295R cells that were treated with 0.4 mM $Bt_2$cAMP for 30 or 60 min. CREB was enriched by 2.6-fold at region A (-1779/-1596) and 15-fold at region D (-325/-214) in cells treated with $Bt_2$cAMP for 30 min (Figure 3.6B). One h exposure to $Bt_2$cAMP increased CREB recruitment to region A by 4.9-fold. Region A is also shown in the agarose gel in the panel B inset. $Bt_2$cAMP had no significant effect on CREB binding in the other three regions (B, C, and E) of the ASAH1 promoter that were examined.

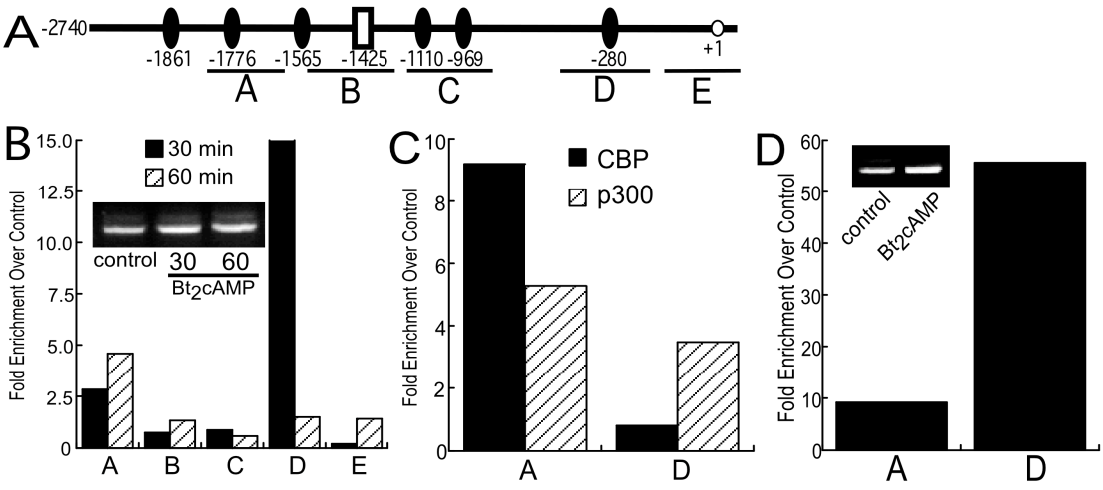



**Figure 3.6.** *CREB, CBP, and p300 bind to the ASAH1 promoter in vivo.* **A.** Diagram of the promoter regions amplified by each primer set used for ChIP assay. **B.** H295R cells were treated for 30 or 60 min with 0.4 mM $Bt_2$cAMP**,** cross-linked with 1% formaldehyde, and the sheared chromatin immunoprecipitated with antibodies against anti-phospho-$Ser^{133}$-CREB. **C.** Chromatin isolated from cells treated for 60 min with 0.4 mM $Bt_2$cAMP was immunoprecipitated with antibodies against anti-CBP or anti-p300 and recruitment to region A or D of the ASAH1 promoter assessed by qPCR. **D.** H295R cells were treated for 30 min with 0.4 mM $Bt_2$cAMP and the isolated chromatin immunoprecipitated with an antibody against trimethyl-H3K4. In panels B, C, and D, DNA purified was quantified by real time PCR and normalized to the ΔCt values of input DNA. Data is expressed as fold change over untreated control. Representative agarose gels of the PCR products obtained for region A are shown as insets to panel B (control, 30, and 60 min $Bt_2$cAMP treatment) and panel D (control and 30 min $Bt_2$cAMP treatment).

I also assessed the effect of cAMP signaling on coactivator association with the regions (A and D) of the ASAH1 promoter that exhibited enriched CREB binding. Both CBP and p300 were recruited to region A in cells exposed to $Bt_2$cAMP for 60 min (Figure 3.6C). Finally, because activated gene transcription is associated with the increased

presence of specific histone modifications, I determined the effect of $Bt_2$cAMP on the amount of trimethylated H3K4, a modification associated with active transcription (411), in regions A and D of the ASAH1 promoter. As shown in Figure 3.6D (region A shown in agarose gel inset), $Bt_2$cAMP evoked an increase in the trimethylation of H3K4, indicating that $Bt_2$cAMP-stimulated CREB binding occurs concomitantly with histone H3 modification.

### *3.3.6. $Bt_2$cAMP increases ASAH1 protein expression and enzymatic activity*

Because $Bt_2$cAMP increased ASAH1 mRNA levels, I sought to investigate if this effect translates into an increase in protein expression. Western blotting analysis in H295R cells treated for 24, 48, or 72 h with $Bt_2$cAMP was carried out. As shown in

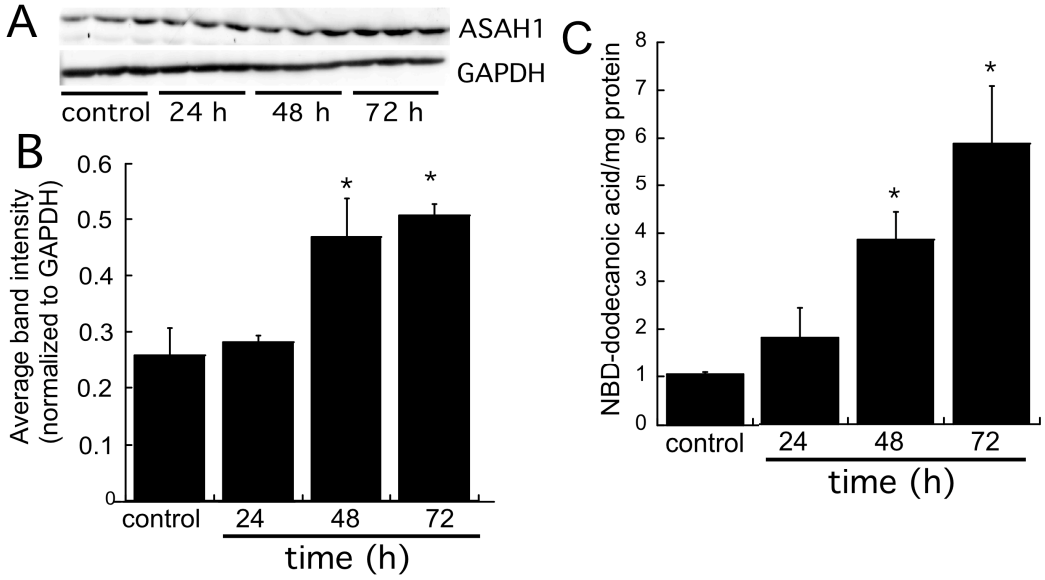



**Figure 3.7.** *$Bt_2$cAMP increases ASAH1 protein expression and enzymatic activity.* **A.** Representative western blot of H295R cells treated for 24, 48, or 72 h with 0.4 mM $Bt_2$cAMP. Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-ASAH1 or anti-GAPDH antibodies. **B.** Graphical representation of the normalized band intensities shown in A. **C.** H295R cells were treated for 24, 48, or 72 h with 0.4 mM $Bt_2$cAMP and cell lysates were isolated and incubated (100μL of lysate per reaction) with 2μL of 1 mM NBD-12-Cer in acetate buffer (pH 4.5) for 2 h at 37°C. Lipids were extracted and spotted on TLC plates. Plates were developed in chloroform:methanol:25% $NH_4OH$ (90:20:0.5, v/v/v) and visualized. NBD-dodecanoic acid formation was quantified and normalized to the protein content of each sample. Data shown is representative of 2 separate experiments each done in duplicate. (*) indicates a statistically significant difference compared to untreated controls ($p < 0.05$).

Figure 3.7A and B, $Bt_2$cAMP significantly increased ASAH1 protein levels by 1.81- and 1.96-fold after 48 and 72 h treatment, respectively. In addition, to determine if the increase in protein expression was functionally significant, ASAH1 activity in $Bt_2$cAMP-treated H295R cells was quantified. As shown in figure 3.7C, $Bt_2$cAMP significantly increased total ceramidase activity by 3.7- and 5.6-fold after 48 and 72 h.

### 3.4. Discussion

ACTH/cAMP signaling regulates cortisol biosynthesis in the human adrenal cortex by chronically stimulating the transcription of multiple genes required for cholesterol metabolism and transport (27). In addition, ACTH acutely increases sphingolipid metabolism in H295R cells, resulting in changes in the cellular concentrations of bioactive sphingolipids that regulate steroidogenic gene transcription (75). In this Chapter, I show that ACTH/cAMP induces ASAH1 gene transcription by promoting the binding of CREB to multiple regions on the ASAH1 promoter.

$Bt_2$cAMP differentially regulates ASAH genes (Figure 3.1A) and the transcription of the ASAH1 gene is modulated by $Bt_2$cAMP in a time-dependent manner with maximal induction of the gene at the 18 h time point (Figure 3.1B). Interestingly, $Bt_2$cAMP acutely reduces ASAH1 mRNA expression within 1 h. These data indicate that ACTH/cAMP signaling evokes a biphasic pattern of ASAH1 mRNA expression, with an initial decrease followed by transcriptional induction. The rapid decrease in the mRNA expression of ASAH1 suggests that cAMP may regulate the stability of ASAH1 transcripts and possibly promote the acute degradation of ASAH1 mRNAs. Regulation of RNA stability is a fundamental mechanism of gene regulation.

MicroRNAs (miRNA) and 3'UTR AU-rich elements (AURE) are two types of regulatory molecules capable of destabilizing transcripts (412-414). miRNAs are single-stranded RNA molecules that downregulate gene expression by binding to complementary regions of mRNA molecules and either targeting them for degradation or blocking the assembly of the translational machinery (414). AUREs contain clustered or distributed AUUUA pentameric motifs or an U-rich sequence where interacting proteins bind to either stabilize (e.g. HuR) or destabilize (e.g. TTP, KSRP) the transcript by signal transduction pathways (415-416). Of note, these regulatory elements have been shown to regulate the expression of genes involved in steroidogenesis including SPHK1 (417)

and StAR (418). Multiple miRNA target sites and 3'UTR AU-rich regions are present on the ASAH1 mRNA (Lucki and Sewer, unpublished observation). Thus, it is possible that these regulatory mechanisms are involved in the observed acute ASAH1 mRNA decrease induced by $Bt_2$cAMP. Interestingly, a similar acute decrease in mRNA expression was also observed for ASAH2 and ACER3 in response to ACTH and $Bt_2$cAMP, suggesting that a similar regulatory mechanism may be involved (Lucki and Sewer, unpublished observation).

Luciferase assays using deletion constructs of the ASAH1 promoter identified a role for CREB in increased reporter gene activity that was localized to the region encompassing -2740 and -906 base pairs upstream of the transcription start site (Figure 3.2). ChIP (Figure 3.6) and EMSA (Figure 3.5) studies support a role for cAMP in stimulating the association of CREB with the ASAH1 promoter. ChIP experiments (Figure 3.6) revealed that CREB is enriched at region -325/-214 (region D) of the ASAH1 promoter, however no stimulatory effect of CREB overexpression on reporter gene activity was observed when cells were transfected with the pGL3-ASAH1(-496) plasmid that contains the –325/-214 region (Figure 3.2). It is possible that although $Bt_2$cAMP promotes CREB recruitment to the endogenous promoter, an increase in transcription requires the coordinate binding of CREB to multiple regions of the ASAH1 promoter. It is equally likely that cAMP signaling increases the binding of additional transcription factors in conjunction with CREB to the promoter. Nonetheless, RNAi (Figure 3.4) and the use of a dominant-negative CREB expression plasmid (Figure 3.3) confirmed the integral role of CREB in conferring increased gene expression in response to $Bt_2$cAMP. Trimethylation of H3K4 and the recruitment of CBP and p300 to both the distal (region A) and proximal (region D) regions of the promoter further support a role for CREB in activating ASAH1 gene transcription.

Functional promoter characterization has been reported for some of the genes encoding sphingolipid-metabolizing enzymes, including SGPP2 (222), subunit 2 of SPT (SPTLC2) (419), ganglioside GM3 synthase (420), ceramide glucosyltransferase (421), and ASAH2 (422). However, few studies have identified the transcription factors involved in the regulation of these genes. Sobue *et al.* (221) reported a NGF-mediated induction of the SPHK1 gene via binding of Sp1 to a specific 5' region of the promoter and Mechtcheriakova *et al.* (222) demonstrated that NF-κB is necessary for induction of the SGPP2 gene in response to inflammatory stimuli.

Although previous studies have defined the structural units of the ASAH1 gene (423-424), to our knowledge there is only one report that investigates the transcriptional regulation of an ASAH gene. Park *et al.* (425) characterized a 1931 base pair region of the murine ASAH1 promoter and demonstrated that Kruppel like factor 6 (KLF6), Sp1, and activator protein 2 (AP2) can bind the promoter *in vitro.* The findings in this Chapter provide functional characterization of the ASAH1 promoter and establish the factors that facilitate ACTH/cAMP-dependent ASAH1 gene expression.

I also show that the effect of ACTH/cAMP signaling on ASAH1 transcription results in a significant increase in protein expression (Figure 3.7). Increased protein concentration is, in turn, concomitant with an upregulation in ceramidase enzymatic activity. These data, coupled with previous findings from our laboratory establishing the role of ACTH/cAMP signaling in acutely modulating sphingolipid metabolism (75), demonstrate that activation of the ACTH signaling pathway elicits two temporally distinct effects on sphingolipid metabolism: a rapid response and a chronic, transcriptional response. In addition, these data supports a role for ASAH1 as an important enzyme for the regulation of sphingolipid metabolism in response to ACTH/cAMP signaling that consequently modulates cortisol biosynthesis.

Our group has previously demonstrated that ACTH promotes rapid changes in sphingolipid intracellular concentrations, including a decrease in Cer (47). Thus, my data indicating an ACTH-regulated transcription and activity of ASAH1 is in agreement with such previously finding and suggests that ACTH signaling is directly regulating intracellular SPH and S1P concentrations. Given that SPH is an antagonist ligand for SF-1 and S1P promotes CYP17A1 expression, it is tempting to speculate that the transcriptional regulation of the ASAH1 gene by ACTH/cAMP signaling is part of a regulatory mechanism through which ACTH modulates cortisol production. In summary, this Chapter presents data that supports CREB as a central regulator of ASAH1 gene transcription and demonstrate that activation of the cAMP signaling pathway promotes the coordinate interaction of CREB and the coactivators CBP and p300 to multiple regions of the ASAH1 promoter, concomitant with the trimethylation of H3K4.

## CHAPTER 4:

*Acid ceramidase is a global regulator of steroidogenic capacity and adrenocortical gene expression*

### 4.1. Introduction

In the human adrenal cortex, cortisol is synthesized from cholesterol by cytochrome P450 monooxygenase (encoded by CYP11A1, CYP17A1, CYP11B1, and CYP21A2 genes) and 3β-HSDII enzymes in a process primarily regulated by the peptide hormone ACTH (3,27,426-427). In the zonae fasciculata and reticularis, ACTH increases steroid hydroxylase gene expression by activating adenylyl cyclase and consequently increasing intracellular cAMP (27). This second messenger activates PKA, which acutely promotes cholesterol mobilization to the inner mitochondrial membrane and chronically induces the transcription of genes required for steroid hormone production (264,271,278,428). The transcription of most steroidogenic genes is regulated by SF-1, which in response to ACTH signaling binds to target promoters and facilitates the recruitment of coactivator proteins (264,266,429-432). Further, additional transcription regulators, including β-catenin (433-434), DAX-1 (435-436), and the NR4A family of transcription factors (Nur77, Nurr1, and Nor-1) (25,437-439) are equally important for maintaining optimal transcriptional output.

Sphingolipids have emerged as important second messengers in various signaling transduction pathways (56,89,200,440-444). In steroidogenesis, SPH modulates steroidogenic gene transcription by serving as an antagonist for SF-1 (298). Previous work from our group has demonstrated that SPH is bound to the receptor under basal conditions and that $Bt_2$cAMP stimulation promotes SPH displacement from the receptor ligand-binding pocket. SPH binding to SF-1 antagonizes the ability of $Bt_2$cAMP to activate CYP17A1 gene transcription and stimulate DHEA production (47). Silencing the expression of the SPH-generating enzyme ASAH1 mimics $Bt_2$cAMP-

stimulated CYP17A1 transcription (298), which supports a role for this enzyme in regulating SF-1 function and steroidogenic gene transcription. In many respects, steroid hormone biosynthesis and sphingolipid metabolism have a reciprocal relationship (445). In H295R cells, ACTH stimulates sphingolipid metabolism by rapidly promoting the catabolism of SM, Cer, and SPH (75). ACTH/cAMP signaling acutely increases the enzymatic activities of SPHK (75,115) and ASAH1 (Chapter Three) in H295R cells. Further, as described in Chapter Three, CREB is an essential transcriptional regulator of the ASAH1 gene in H295R cells.

Ceramidases are a family of hydrolases that catalyze the degradation of Cer into SPH and a free fatty acid. ASAH1 is a glycoprotein processed from a 55 kDa precursor via autoproteolytic cleavage (107) into a mature heterodimeric enzyme formed by an $\alpha$ (13 kDa) and $\beta$ (40 kDa) subunits (108). Because Cer degradation is the only source of cellular SPH (104), these enzymes are not only essential for limiting Cer-mediated signaling but also for controlling the cellular functions of SPH and S1P (83,105-106). In mouse, ASAH1 is expressed early during embryogenesis, with targeted disruption of the ASAH1 gene resulting in embryonic lethality (109). Moreover, ASAH1 overexpression has been reported in various human cancers (110-113) and a genetic deficiency in ASAH1 catalytic activity causes the lysosomal sphingolipid storage disorder, Farber's disease (114).

In order to determine the functional significance of Cer metabolism in adrenocortical steroidogenesis, an H295R stable cell line that expresses ASAH1-shRNA (short hairpin RNA) in a tetracycline (tet)-regulated manner was generated. Experiments in this Chapter show that suppression of ASAH1 protein expression results in global changes in gene expression, including the induction of the steroidogenic genes CYP17A1, CYP11A1, CYP21A2, CYP11B, StAR, TSPO, HSL, and Mc2R. Consistent with increased steroidogenic gene expression, these cells exhibit a higher capacity to

secrete cortisol and DHEA, both basally and in response to activation of the ACTH signaling pathway. Furthermore, suppression of ASAH1 alters the amount of several sphingolipid species, including a decrease in $C_{16}$-sphingolipids and a shift in the acyl-chain composition of Cer, SM, and glycosphingolipid subspecies. Intriguingly, ASAH1 knockdown leads to a decrease in cell proliferation with a concomitant reduction in the protein levels of β-catenin, proliferating cell nuclear antigen (PCNA), and cyclin B2.

## 4.2. Materials and Methods

### *4.2.1. Cell Culture*

H295R adrenocortical cells were cultured as described in Chapter Two. H295R ASAH1$^{KD}$ (ASAH1-knockdown) cells were cultured in the same medium as the parental cell line with the addition of 1 μg/mL blasticidin and 50 μg/mL zeocin.

### *4.2.2. Generation of the H295R ASAH1-knockdown (ASAH1$^{KD}$) stable cell line*

H295R cells expressing tetracycline (tet)-inducible ASAH1shRNA were generated using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen) following the manufacturer's instructions. First, the tet repressor (TetR) expression plasmid (pcDNA6-TR, Invitrogen) was stably transfected into H295R cells using GeneJuice (EMD Biosciences). Each clone was selected using 1 μg/mL blasticidin and TetR expression was determined by western blotting using an anti-TetR antibody (Sigma). The clone that expressed the highest TetR protein (H295R-TetR cells) was transfected with the pENTR/H1/TO-ASAH1shRNA vector. To construct an inducible vector for ASAH1 shRNA, the following sequences were cloned into pENTR/H1/TO: 5'-ACC GCA CCA ATG CTA AAG GTT ATA GTG AAC GAA TTC ACT ATA ACC TTT AGC ATT GGT G-3' and 5'-AAA ACA CCA ATG CTA AAG GTT ATA GTG AAT TCG TTC ACT ATA ACC TTT AGC ATT GGT GC-3', corresponding to positions 221 to 246 of the ASAH1 mRNA sequence (NM_004315). H295R-TetR cells were stably transfected with the constructed pENTR/H1/TO-ASAH1shRNA expression vector or the control vector using GeneJuice (EMD Biosciences), and cell clones were selected using 50 μg/mL zeocin. Clones were treated with 5 μg/mL tet for 96 h and suppression of ASAH1 protein levels in each clone was confirmed by western blotting using an anti-ASAH1 antibody (HPA005468, Sigma).

### *4.2.3. DNA Microarray*

H295R wild type (WT) or tet-treated (5 μg/mL for 72 h) ASAH1$^{KD}$ cells were sub-cultured into 100-mm dishes and treated with 0.4 mM $Bt_2$cAMP for 18 h. Total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Valencia, CA) and gene expression profiling was done by Phalanx Biotech Group, Inc. (Palo Alto, CA) using the Human Whole Genome OneArray™ Microarray (HOA_004).

### *4.2.4. RNA isolation and real-time RT-PCR (qRT-PCR)*

H295R WT or ASAH1$^{KD}$ cells were sub-cultured into 12-well plates and shRNA cells treated with 5 μg/mL tet for 48 - 72 h. In some experiments, cells were stimulated with 0.4 mM $Bt_2$cAMP for 18 h prior to RNA extraction. Total RNA was isolated using Isol-RNA Lysis Reagent (5 Prime, Inc., Gaithersburg, MD) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Scientific Inc., Waltham, MA) and the primer sets listed in Table 4.1. Gene expression was normalized to β-actin mRNA content and calculated using the delta-delta cycle threshold (ΔΔCT) method.

### *4.2.5. Western Blotting*

H295R WT or ASAH1$^{KD}$ (5 μg/mL tet for 0 to 120 h) cells were harvested into RIPA buffer containing protease inhibitors. Cells were then lysed by sonication (one 2 sec burst) followed by incubation on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (30 μg of protein) were resolved on 8% SDS-PAGE gels and transferred to PVDF membranes (Milipore, Temecula, CA). Blots were probed with

**Table 4.1.** Primer sets used in qRT-PCR experiments.

| Gene | Forward (5'- 3') | Reverse (5'- 3') |
|---|---|---|
| **β-Actin** | ACGGCTCCGGCATGTGCAAG | TGACGATGCCGTGCTGCATG |
| **ASAH1** | GCACAAGTTATGAAGGAAGCCAAG | TCCAATGATTCCTTTCTGTCTCG |
| **ASAH2** | GCATCAACACAGGAGAGTC | GGAGGCAGAGGCATAGAG |
| **ACER3** | ATCCGCCTGGTCTTCATC | CTCCTTATTGCTGGTCTTCC |
| **SPHK1** | CTGGCAGCTTCCTTGAACCAT | TGTGCAGAGACAGCAGGTTCA |
| **SPHK2** | CCAGTGTTGGAGAGCTGAAGGT | GTCCATTCATCTGCTGGTCCTC |
| **SPTLC1** | AGTCCCTTTCTCCAGCCTTTC | TTCCACCGTGACCACAACC |
| **SPTLC2** | CAAGAAGAAATACAAGGCATAC | CCATCATAACATCCACATCC |
| **CerS1** | CCTTCTACTTCTTCTTCAATGC | TCGGCTGTGTCATACTCC |
| **CerS2** | GTCATCCTGCCCTTCTGG | CACTGCTGGCATCTTCTACC |
| **CerS3** | TCATACATCTTCCTCAACCTACAG | GCCTCTTCTTCTTCCTCTTCC |
| **CerS5** | ATGGTGGCTCCTCAATGG | AGGTGGTCACATCTTCTTCC |
| **CerS6** | GCTGACGAGGTTCTGTGAG | AGTTGTGAGTGGCTGATAGG |
| **ELOVL1** | GTCTACAACTTCTCACTGGTGGC | AAGTGCCTCAGGGCTGTTGGAA |
| **ELOVL2** | TCCACTTGGGAAGGAGGCTACA | CCAGGAACTCTACTGATTTGGAG |
| **ELOVL4** | CCGAGAACCTTTTCAGATGCGTC | AATCCACACTCTGGCAAATATAG |
| **ELOVL5** | ACGTCTACCACCATGCCTCGAT | TGGAAGGGACTGACGACAAACC |
| **ELOVL6** | CCATCCAATGGATGCAGGAAAAC | CCAGAGCACTAATGGCTTCCTC |
| **ELOVL7** | CCTACTATGGACTTTCTGCATTGG | GAACTGGCTTATGTGGATGGCG |
| **CYP17A1** | CTCTTGCTGCTTCACCTA | TCAAGGAGATGACATTGGTT |
| **CYP11A1** | CGTGGAGTCGGTTTATGTC | CTCTGGTAATACTGGTGATAGG |
| **CYP11B** | ACGGCGACAACTGTATCC | AGAGCGTCATCAGCAAGG |
| **CYP21A2** | TGTGGAACTGGTGGAAGC | GGTGGAGCCTGTAGATGG |
| **3β-HSD** | CCAGTAGCATAGAGGTAGCC | TCAGATTCCACCCGTTAGC |
| **NR5A1** | GGAGTTTGTCTGCCTCAAGTTCA | CGTCTTTCACCAGGATGTGGTT |
| **NR5A2** | TACCGACAAGTGGTACATGGAA | CGGCTTGTGATGCTATTATGGA |
| **NR4A1** | GGACAACGCTTCATGCCAGCAT | CCTTGTTAGCCAGGCAGATGTAC |
| **NR4A2** | AAACTGCCCAGTGGACAAGCGT | GCTCTTCGGTTTCGAGGGCAAA |
| **NR4A3** | ACTGCCCAGTAGACAAGAGACG | GTTTGGAAGGCAGACGACCTCT |
| **NR0B1** | CCAAATGCTGGAGTCTGAACATC | CCCACTGGAGTCCCTGAATGTA |
| **NR0B2** | TGCCTGAAAGGGACCATCCTCT | GTTCCAGGACTTCACACAGCAC |
| **StAR** | GCTCTCTACTCGGTTCTC | GCTGACTCTCCTTCTTCC |
| **TSPO** | GCAGATTCCGTGATTACAGTG | TCCTCCTCGTCGTCATCG |
| **HSL** | CACTACAAACGCAACGAGAC | CCAGAGACGATAGCACTTCC |
| **Mc2R** | GTGGTGCTTACGGTCATCTGGA | AGGCACAGGATGAAGACCAGCA |
| **PDE4B** | TAGTCAGCCTCCTGTCTCCAGA | GAAGCCATCTCACTGACAGACC |
| **SGPP2** | ACTCCTCATCGTCCTCAC | CAGAATCCTATGGTCACTCC |
| **nSMase 1** | GTGCTCAACGCCTATGTG | GTCTGCCTTCTTGGATGTG |
| **nSMase 2** | ACTGCTCCTCTGACGACAAG | CCACGGCTTCTCCTCACC |
| **SGMS2** | ACTCTACCTGTGCCTGGAATG | AATAAGTCAGTGTCAGCGTAACC |
| **aSMase 1** | TTGTAGCCAGGTATGAGAAC | GCCGATGTAGGTAGTTGC |
| **CERK** | ACCACTGACATCATCGTTACTG | GCACCTCGCTGAACATACC |
| **DEGS1** | GTTTGGAATGTTTGCTAATC | GAGAGGCTGAAGAATAACC |

multiple antibodies and expression was detected using an ECF western blotting reagent kit (GE Healthcare, Piscataway, NJ) and visualized by scanning the blots on a VersaDoc 4000 imager (Bio-Rad, Hercules, CA). Protein amounts were determined using the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL).

### *4.2.6. Analysis of sphingolipid amounts*

H295R WT or ASAH1$^{KD}$ cells (pre-treated with 5 μg/mL tet for 96 h) were sub-cultured into 100-mm dishes and sphingolipid amounts in whole cells were analyzed by liquid chromatography, electrospray ionization, tandem mass spectrometry (LC-ESI-MS/MS) as described previously (446-447).

### *4.2.7. Cortisol and DHEA enzyme-linked immune assays (EIA)*

H295R WT or ASAH1$^{KD}$ cells (pre-treated with 5 μg/mL tet for 72 h) were sub-cultured into 12-well plates and treated with 0.4 mM $Bt_2$cAMP for 48 h prior to media collection. Cortisol and DHEA released into the media was determined in triplicate against cortisol or DHEA standards made up in DMEM/F12 medium using a 96-well plate enzyme-linked immune DHEA or cortisol assay (Assay Designs, Inc., Ann Arbor, MI). Steroid hormone amounts were normalized to the total cellular protein content, as determined using the BCA protein assay (Pierce).

### *4.2.8. Quantification of intracellular cAMP*

H295R WT or ASAH1$^{KD}$ cells (pre-treated with 5 μg/mL tet for 96 h) were cultured into 12-well plates and treated with 50 nM ACTH for 5 min. Cells were lysed in 0.1 M HCl for 20 min at room temperature, collected by scraping, and centrifuged at 4,000 rpm for 10 min. The supernatant was collected, acetylated, and used in a direct

cyclic AMP Correlate-EIA™ (Assay Designs, Inc.) following the manufacturer's instructions. Data were normalized to the total protein amount of each sample and is expressed as picomoles per milligram of total protein.

### *4.2.9. Chromatin Immunoprecipitation (ChIP)*

H295R WT or ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 96 h) cells were sub-cultured into 150-mm dishes and ChIP assays were performed as described previously (158). The purified chromatin solutions were pre-cleared and immunoprecipitated using 3 μg of anti-acetyl-histone H3 (06-599, Millipore) or 3 μg rabbit anti-IgG (Santa Cruz) antibodies and 30 μL protein A/G Plus agarose (Santa Cruz). Quantitative PCR was carried out using 20% of output, 5% input (diluted 1:4), the ABsolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific), and the primer sets listed in Table 4.2.

**Table 4.2**. Primer sets used in ChIP studies.

| Gene | Forward (5'- 3') | Reverse (5'- 3') |
|---|---|---|
| **StAR** | GCAGTGTGAGGCAATCGCTCT | TGTTTCCTGGCAAATGTGGCA |
| **Mc2R** | TTGCCCAGAAAGTTCCTGCTT | TTCTCCTGCTTGTGGTTAAGG |
| **CYP17A1** | GGCTGGGCTCCAGGAGAATCTTTCTTCCAC | GGCAGGCAAGATAGACAGCAGTGGAGTAG |
| **DAX-1** | TCCTGCTTTTAAAGAGCACCCGCCCCT | CGGCGCCCGTAGCCCAGTTCT |
| **NR4A2** | GATCAGCTTGGACTCCCTTGAAAGT | CATTCCAGAAATTGGCATCTTTTGA |

### *4.2.10. Cell Proliferation*

For quantitative proliferative assays, H295R wild type (WT), H295R-TetR, and ASAH1$^{KD}$ cells were seeded in 96-well plates (5 x $10^3$ cells/well). H295R-TetR and ASAH1$^{KD}$ cells were treated for 96 h with 5 μg/mL tet. After treatment, the cultures were incubated for an additional 6 h in the presence of 5-bromo-2-deoxyuridine (BrdU; 10 μM). Cell proliferation was assayed by BrdU incorporation measurements with an ELISA kit (Roche Applied Science, Indianapolis, IN).

### *4.2.11. Statistical Analysis*

One-way ANOVA, Tukey-Kramer multiple comparison, and unpaired student t-tests were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Significant differences from a compared value were defined as $p < 0.05$ and denoted by asterisks (*) or carats (^). Hierarchical clustering analysis of DNA microarray studies was done by performing observation and variable tree computation using complete linkage clustering and correlation distance matrix with robust center scale normalization.

## 4.3. Results

### *4.3.1. Characterization of the H295R ASAH1$^{KD}$ stable cell line*

To characterize the functional significance of ASAH1 expression in regulating the steroidogenic capacity of adrenocortical cells, H295R cells were stably transfected with the pENTR/H1/TO vector containing a sequence for an ASAH1 shRNA. As shown in Figure 4.1A, ASAH1 mRNA levels were reduced by 68% in tet-treated cells (5 μg/mL tet for 48 h). Importantly, ASAH2 and ACER3 mRNA expression remained unchanged. ASAH1 protein levels are reduced by 52% and 81% after 72 h and 96 h tet treatment,

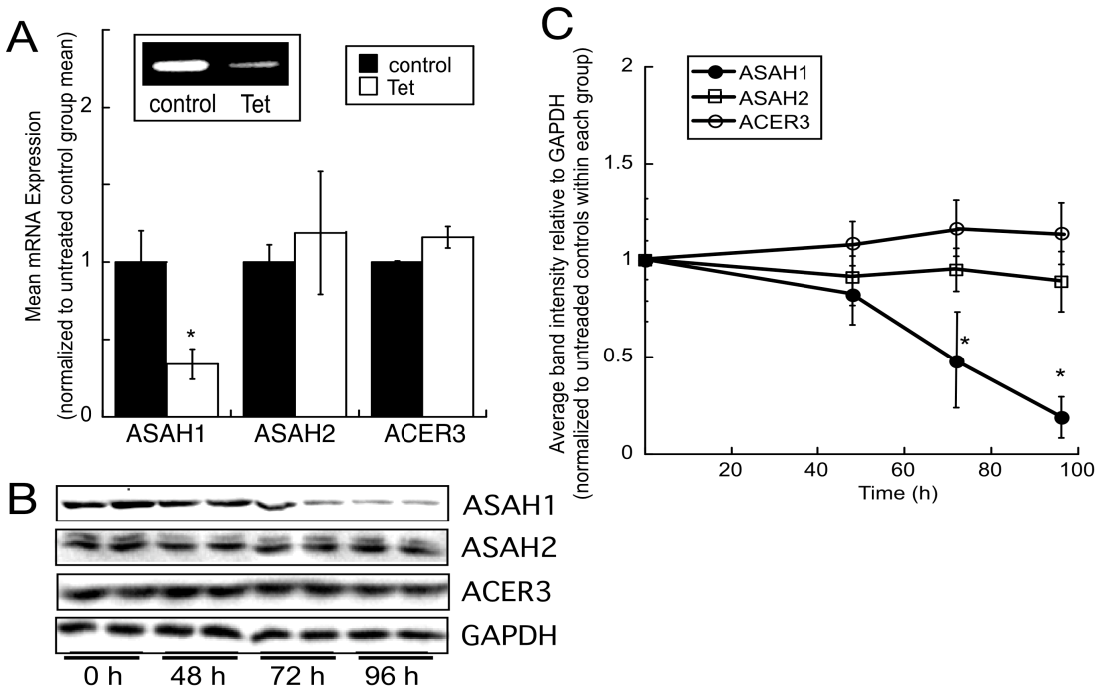



**Figure 4.1.** *Characterization of the H295R ASAH1$^{KD}$ cell line.* **A**. ASAH1$^{KD}$ cells were treated with 5 μg/mL tetracycline (tet) for 48 h, total RNA was harvested, and ASAH1, ASAH2, and ACER3 mRNA levels were quantified by qRT-PCR and was normalized to the mRNA expression of β-actin. *Inset:* representative agarose gel of qRT-PCR products from ASAH1 mRNA transcripts. Data is graphed as mean +/- SEM of three separate experiments, each done in triplicate. Asterisks (*) indicate statistically significant differences from untreated controls ($p < 0.05$). **B**. ASAH1$^{KD}$ cells were treated with 5 μg/mL tet for 48, 72, or 96 h, cell lysates were harvested, and separated by SDS-PAGE followed by western blotting using antibodies against ASAH1, ASAH2, ACER3, and GAPDH. **C.** Densitometric analysis of western blots of ASAH1, ASAH2, and ACER3 protein expression in, normalized to GAPDH protein content in ASAH1$^{KD}$ cells treated for 0 to 96 h with 5 μg/mL tet. Data graphed represent the mean ± STDEV of 3 separate experiments, each done in duplicate. Asterisks (*) denote statistically significant difference ($p < 0.05$) versus untreated controls within each group.

respectively (Figures 4.1B and 4.1C). Similar levels of ASAH1 suppression were observed in another clone (data not shown).

### *4.3.2. ASAH1 suppression alters steroidogenic gene expression*

Because SPH is an antagonist for SF-1 and transient ASAH1 silencing induces CYP17A1 transcription (47), the effect of reduced ASAH1 expression on global gene expression was determined. ASAH1 knockdown altered the expression of multiple classes of genes, including cell cycle and transcriptional regulators (data not shown). In addition, as shown in Figure 4.2, reduction of ASAH1 expression markedly changed the expression of genes required for steroid hormone metabolism. Real-time RT-PCR revealed that ASAH1 knockdown induced CYP11B and CYP21A2 transcription by 2.1- and 5.6-fold, respectively, while increased the expression of CYP17A1 by 4.8-fold (Figure 4.2B). In addition to increasing the mRNA expression of CYP11A1 by 1.6-fold, ASAH1 suppression also potentiated $Bt_2$cAMP-induced CYP11A1 mRNA expression (Figure 4.2B). Intriguingly, ASAH1$^{KD}$ cells displayed significantly increased expression of genes involved in the uptake, de-esterification, and transport of cholesterol. As shown in Figure 4.2C, StAR, TSPO, and HSL expression was induced by 2.0-fold in ASAH1$^{KD}$ cells. Moreover, mRNA expression of SR-BI, which mediates HDL cholesteryl ester delivery into adrenal cells (448), was also upregulated by 6.8-fold (Figure 4.2C). Notably, suppression of ASAH1 also enhanced $Bt_2$cAMP-stimulated transcription of StAR, HSL, and SR-BI genes. Finally, mRNA expression of the Mc2R gene, which encodes the cognate receptor for ACTH, was induced by 10.1-fold in ASAH1$^{KD}$ cells (Figure 4.2C). Induced mRNA expression was concomitant with an increase in cellular protein levels of these steroidogenic genes (Figure 4.2D).

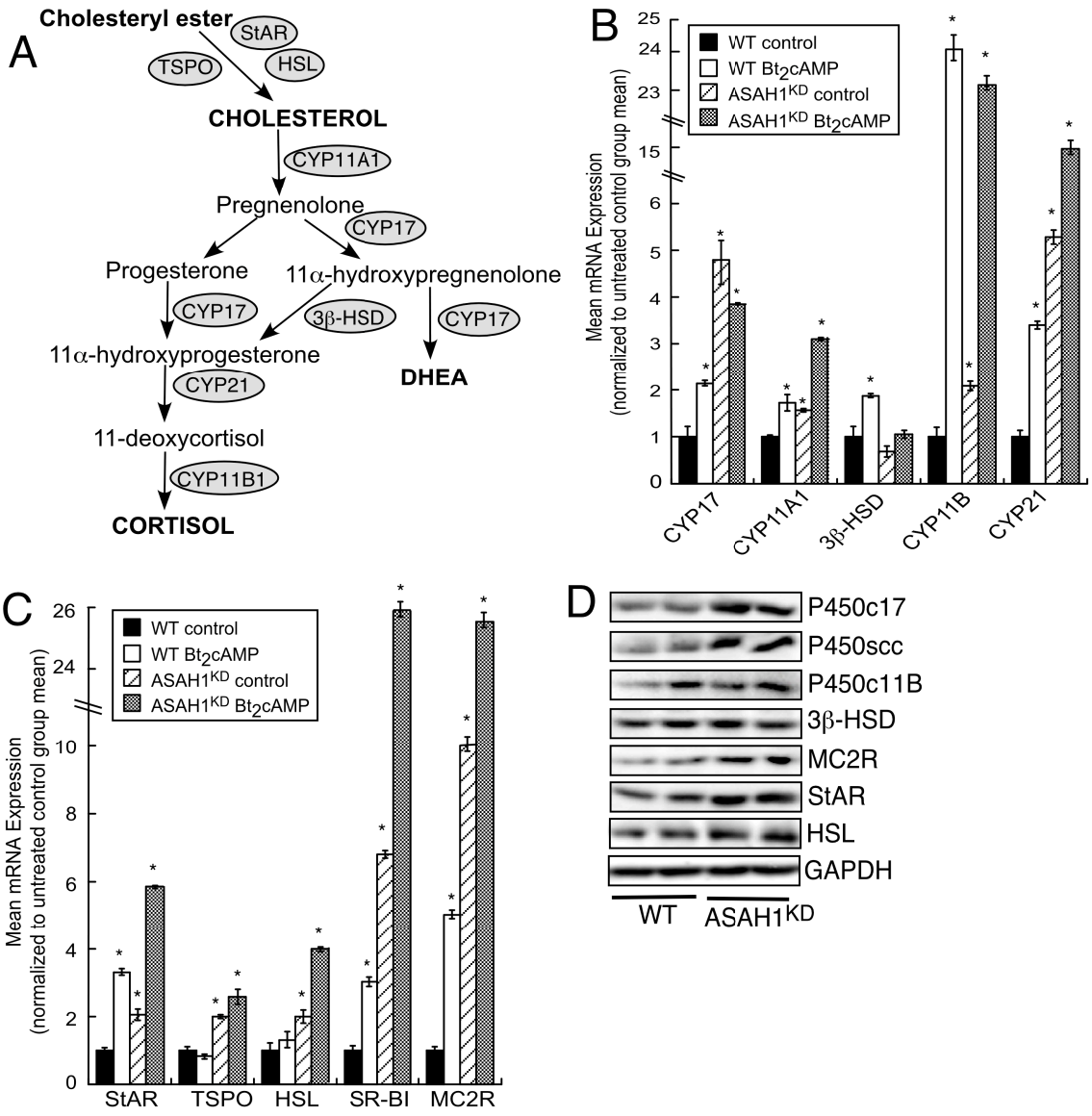



**Figure 4.2.** *ASAH1 knockdown increases the transcription of multiple steroidogenic genes.* **A.** Diagram of the adrenocortical steroid hormone biosynthetic pathway. Circles represent genes that encode proteins that are involved in each step. *Abbreviations:* 18 kDa translocator protein (TSPO), steroidogenic acute regulatory protein (StAR), hormone sensitive lipase (HSL). **B** and **C**. H295R wild type (WT) and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 72 h) cells were treated with 0.4 mM $Bt_2$cAMP for 18 h. Total RNA was isolated and the mRNA levels of various steroidogenic genes were quantified by qRT-PCR and normalized to the mRNA expression of β-actin. Data is graphed as mean +/- SEM of three separate experiments, each done in triplicate. Asterisks (*) indicate statistically significant differences from untreated WT cells ($p < 0.05$). **D**. H295R WT and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 120 h) cell lysates were separated by SDS-PAGE and the protein expression of P450c17 (encoded by CYP17A1), P450scc (CYP11A1), P450c11B (CYP11B), 3β-HSD, Mc2R, StAR, HSL, and GAPDH was quantified by western blotting.

### *4.3.3. Suppression of ASAH1 leads to increased cortisol and DHEA secretion*

Because I observed increased expression of most genes required for steroid hormone production (Figure 4.2), I postulated that ASAH1$^{KD}$ cells have increased steroidogenic capacity. To test this hypothesis, the levels of cortisol and DHEA secreted into the media from controls and $Bt_2$cAMP-treated WT and ASAH1$^{KD}$ cells were quantified. As shown in Figure 4.3A, ASAH1 suppression increased basal cortisol and DHEA secretion by 2.4- and 1.5-fold, respectively, compared to WT cells. Furthermore, downregulation of ASAH1 potentiated the stimulatory effect of $Bt_2$cAMP on steroid hormone secretion (Figure 4.3A).

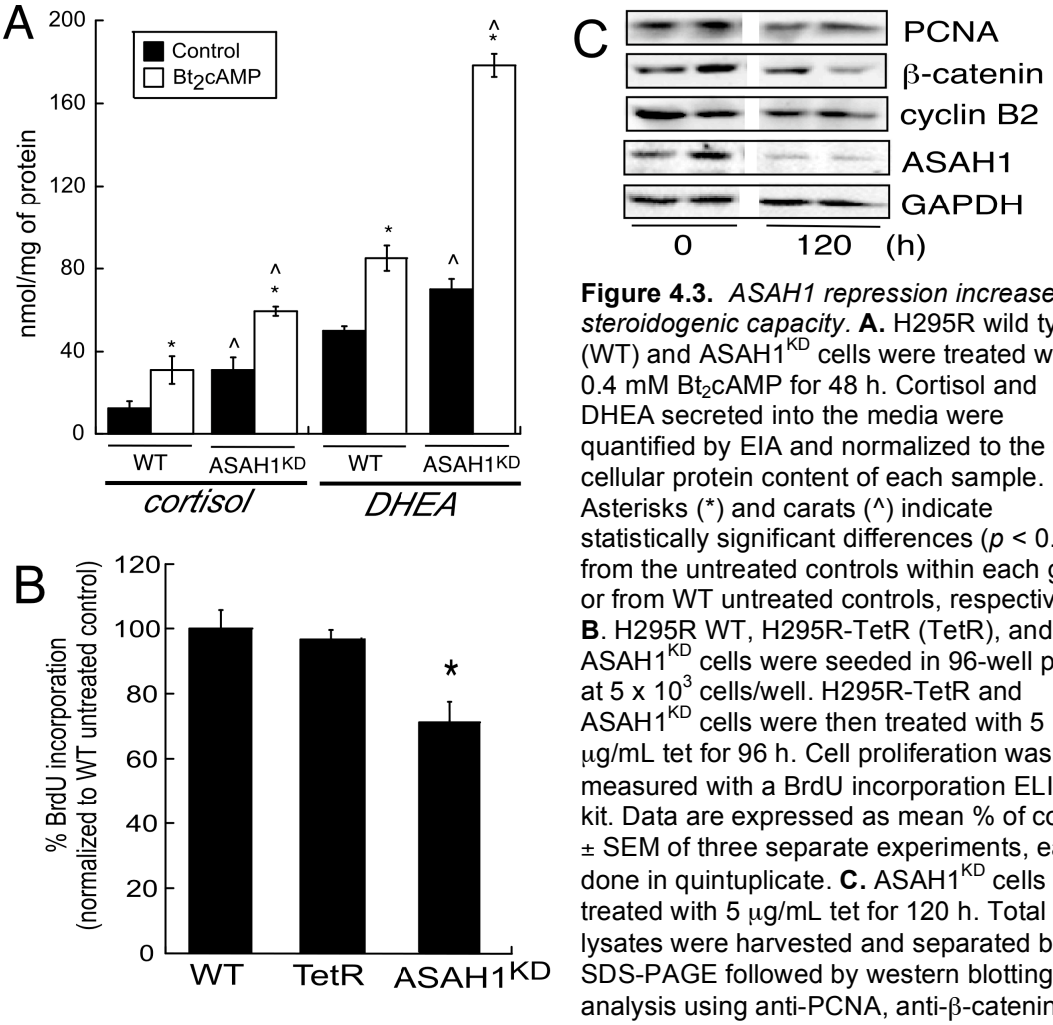



**Figure 4.3.** *ASAH1 repression increases steroidogenic capacity*. **A.** H295R wild type (WT) and ASAH1$^{KD}$ cells were treated with 0.4 mM $Bt_2$cAMP for 48 h. Cortisol and DHEA secreted into the media were quantified by EIA and normalized to the total cellular protein content of each sample. Asterisks (*) and carats (^) indicate statistically significant differences ($p < 0.05$) from the untreated controls within each group or from WT untreated controls, respectively. **B**. H295R WT, H295R-TetR (TetR), and ASAH1$^{KD}$ cells were seeded in 96-well plates at 5 x $10^3$ cells/well. H295R-TetR and ASAH1$^{KD}$ cells were then treated with 5 μg/mL tet for 96 h. Cell proliferation was measured with a BrdU incorporation ELISA kit. Data are expressed as mean % of control ± SEM of three separate experiments, each done in quintuplicate. **C.** ASAH1$^{KD}$ cells were treated with 5 μg/mL tet for 120 h. Total cell lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-PCNA, anti-β-catenin, anti-cyclin B2, anti-ASAH1, or anti-GAPDH antibodies.

### *4.3.4. ASAH1 knockdown decreases cellular proliferation*

To further assess the role of ASAH1 in regulating H295R cell function, the effect of ASAH1 knockdown on cell proliferation was determined. As shown in Figure 4.4B, suppression of ASAH1 expression decreased cell proliferation by 29.1% compared to WT cells. In addition, analysis of various proliferative protein markers revealed that ASAH1 depletion decreased cellular levels of β-catenin, PCNA, and cyclin B2 (Figure 4.3C). Significantly, no change in protein expression was observed in H295R-TetR cells, which express the Tet repressor protein but not ASAH1 shRNA (data not shown). Further, no significant difference in cell viability (determined by MTT assays) was observed among WT, H295R-TetR, and H295R-ASAH1$^{KD}$ cells (data not shown).

### *4.3.5. ASAH1$^{KD}$ cells have altered sphingolipid gene expression and sphingolipid content*

Next, the effect of ASAH1 suppression on the transcription of sphingolipid genes in ASAH1$^{KD}$ cells was examined. As shown in Figure 4.4A, reduced ASAH1 expression increased the mRNA expression of acid SMase 1 (aSMase1), SPHK1, and SPHK2 by 2.3-, 3.3-, and 2.1-fold, respectively. Conversely, the expression of the catalytic subunit 2 of SPT (SPTLC2), the enzyme that catalyzes the rate-limiting step in *de novo* sphingolipid biosynthesis, was significantly decreased by 2.6-fold in ASAH1$^{KD}$ cells (Figure 4.4A). ASAH1$^{KD}$ cells expressed higher levels of CerS1, CerS2, CerS3, and CerS6 mRNA transcripts (Figure 4.4B). These genes encode the family of CerS enzymes that catalyze the *N*-acylation of sphinganine to form dihydroCer (449). Because CerS enzymes use very long-chain fatty acyl-CoAs that are made by the elongation of stearoyl- and palmitoyl-CoAs (449), the expression of the corresponding fatty acyl-CoA

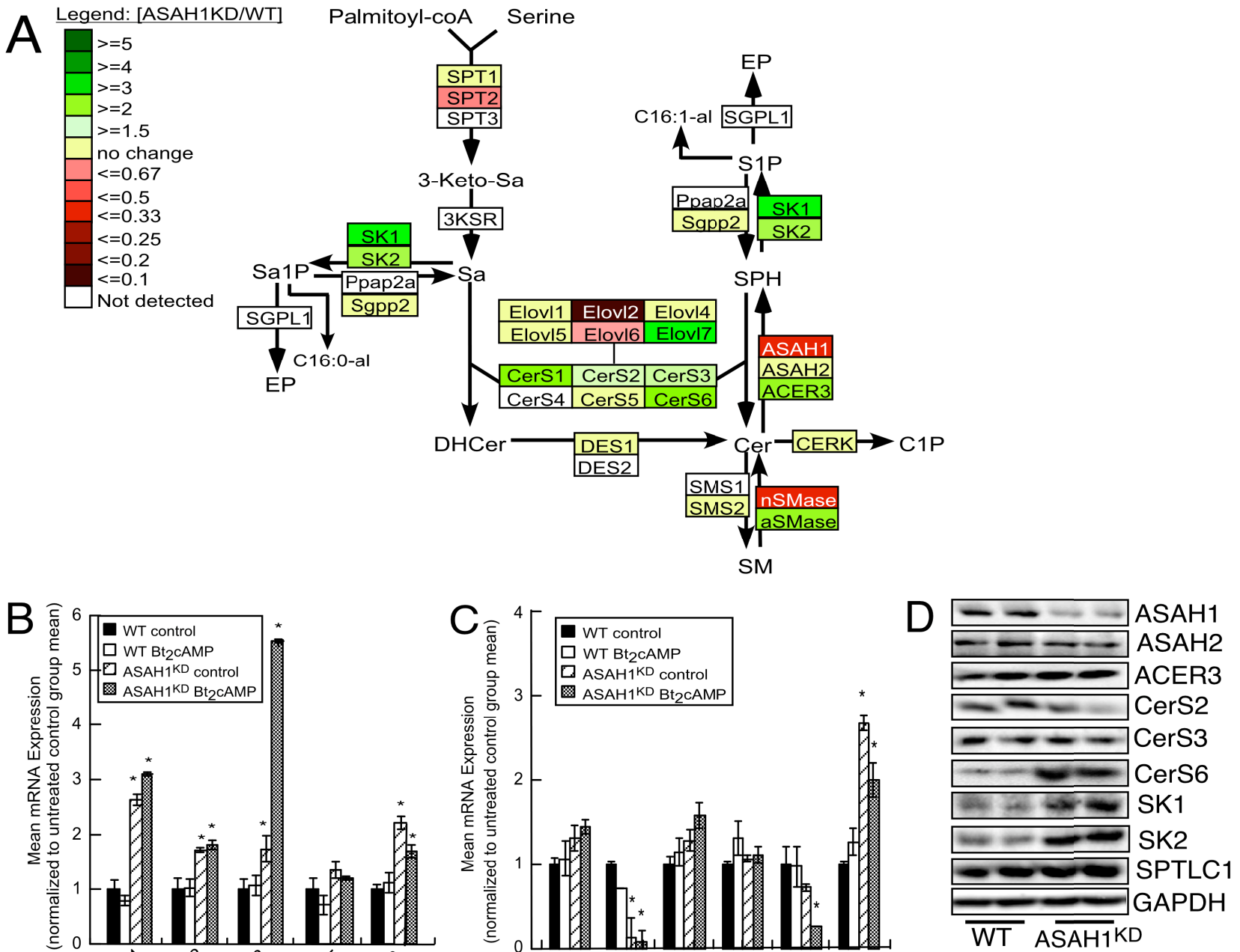

A
Legend: [ASAH1KD/WT]
>=5
>=4
>=3
>=2
>=1.5
no change
<=0.67
<=0.5
<=0.33
<=0.25
<=0.2
<=0.1
Not detected
Palmitoyl-coA
Serine
SPT1
SPT2
SPT3
3-Keto-Sa
3KSR
Sa
Sa1P
SK1
SK2
Ppap2a
Sgpp2
SGPL1
C16:0-al
EP
Elovl1
Elovl2
Elovl4
Elovl5
Elovl6
Elovl7
CerS1
CerS2
CerS3
CerS4
CerS5
CerS6
DHCer
DES1
DES2
Cer
CERK
C1P
ASAH1
ASAH2
ACER3
SPH
S1P
C16:1-al
SGPL1
EP
SMS1
SMS2
nSMase
aSMase
SM
B
Mean mRNA Expression
(normalized to untreated control group mean)
WT control
WT Bt2cAMP
ASAH1KD control
ASAH1KD Bt2cAMP
CerS1
CerS2
CerS3
CerS5
CerS6
C
Mean mRNA Expression
(normalized to untreated control group mean)
WT control
WT Bt2cAMP
ASAH1KD control
ASAH1KD Bt2cAMP
Elovl1
Elovl2
Elovl4
Elovl5
Elovl6
Elovl7
D
ASAH1
ASAH2
ACER3
CerS2
CerS3
CerS6
SK1
SK2
SPTLC1
GAPDH
WT
ASAH1KD

**Fig. 4.4.** *ASAH1 suppression alters the transcription of multiple sphingolipid genes*. **A**. Total RNA from H295R wild type (WT) and ASAH1$^{KD}$ cells (pre-treated with 5 μg/mL tet for 96 h) was isolated as described in Section 4.2. Messenger RNA levels for the indicated sphingolipid genes were determined by qRT-PCR and are displayed as a heat map incorporated into the sphingolipid metabolic pathway. The mean mRNA expression values in ASAH1$^{KD}$ cells range from low (red) to high (green) compared to the mean level of mRNA expression for each gene in H295R WT cells. *Abbreviations:* Serine palmitoyltransferase (represented by 3 SPT genes), 3-ketosphinganine (3-keto-Sa), sphinganine (Sa), 3-keto-Sa reductase (-KSR), sphingosine kinase (SPHK), sphinganine-1-phosphate (Sa1P), Sa1P phosphatase (Ppap2a and Sgpp2), sphingosine-1-phosphate (S1P), S1P lyase (SGPL1), ethanolamine phosphate (EP), hexadecanal (C16:0/1-al), fatty-acyl-CoA elongases (Elovl1-7), Ceramide synthase (CerS), dihydroCeramide (DHCer), DHCer desaturase (DES), Ceramide (Cer), Cer kinase (CERK), Cer-1-phosphate (C1P), sphingomyelin (SM), SM synthase (SMS1-2), sphingomyelinase [SMase, neutral (n) and acid (a)], Ceramidase [acid (ASAH1), neutral (ASAH2), and alkaline (ACER3)], sphingosine (SPH). **B** and **C**. H295R WT and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 72 h) cells were treated with 0.4 mM $Bt_2$cAMP for 18 h. Total RNA was isolated and the mRNA levels of CerS (B) or Elovl (C) genes were quantified by qRT-PCR and normalized to the mRNA expression of β-actin. Data is graphed as mean +/- SEM of three separate experiments, each done in triplicate. Asterisks (*) indicate statistically significant differences from untreated WT cells ($p < 0.05$). **D**. H295R WT and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 120 h) cell lysates were separated by SDS-PAGE and the protein expression of ASAH1, ASAH2, ACER3, CerS2, CerS3, CerS6, SPHK1, SPHK2, SPTLC1, and GAPDH was quantified by western blotting.

elongases (encoded by Elovl genes) was analyzed. The mRNA transcript levels of Elovl2, which catalyzes the elongation of polyunsaturated fatty acyl-CoA of up to 24 carbons (450), were decreased by 7.6-fold in ASAH1$^{KD}$ cells (Figure 4.4C). Conversely, mRNA expression of Elovl7, which encodes a saturated very-long-chain fatty acid elongase (451), was induced by 2.7-fold (Figure 4.4C). Protein expression levels of ASAH1, ASAH2, SPHK1, and SPHK2 mirrored the changes in mRNA expression (Figure 4.4D). While CerS transcripts were increased in ASAH1$^{KD}$ cells, CerS protein levels, with the exception of CerS6, remained unchanged (Figure 4.4D), suggesting multiple levels of regulation of the CerS gene family. Of note, although ACER3 mRNA expression was induced in response to suppression of ASAH1 expression (Figure 4.4A), no statistically significant change in protein level was observed (Figure 4.4D).

These changes in sphingolipid gene expression prompted me to quantify the intracellular amounts of various sphingolipid species in ASAH1$^{KD}$ cells. LC-ESI-MS/MS analysis in WT and ASAH1$^{KD}$ cells revealed that total cellular SPH and S1P amounts were decreased by 30% and 16%, respectively, when ASAH1 was suppressed (Figure 4.5A). Significantly, suppression of ASAH1 expression prevented the acute $Bt_2$cAMP-stimulated sphingolipid turnover that was observed in WT cells (Figure 4.5B) (295). ASAH1 knockdown altered the relative levels of distinct Cer and SM subspecies (Figure 4.5C). The amounts of saturated long-chain ($C_{18-24}$) Cer and unsaturated long-chain ($C_{18:1}$) Cer were proportionally increased while $C_{16}$-Cer and unsaturated very long-chain ($C_{24:1}$ and $C_{26:1}$) Cer were decreased in ASAH1$^{KD}$ cells compared to WT (Figure 4.5C). Similarly, long-chain ($C_{18-22}$), very long-chain ($C_{26}$), and unsaturated long-chain ($C_{18:1}$) SM species were proportionally increased while $C_{16}$-SM was decreased in ASAH1$^{KD}$ cells (Figure 4.5C). Intriguingly, the amounts of LacCer and HexCer were decreased by 40% and 70%, respectively, in response to ASAH1 knockdown, with decreases in specific

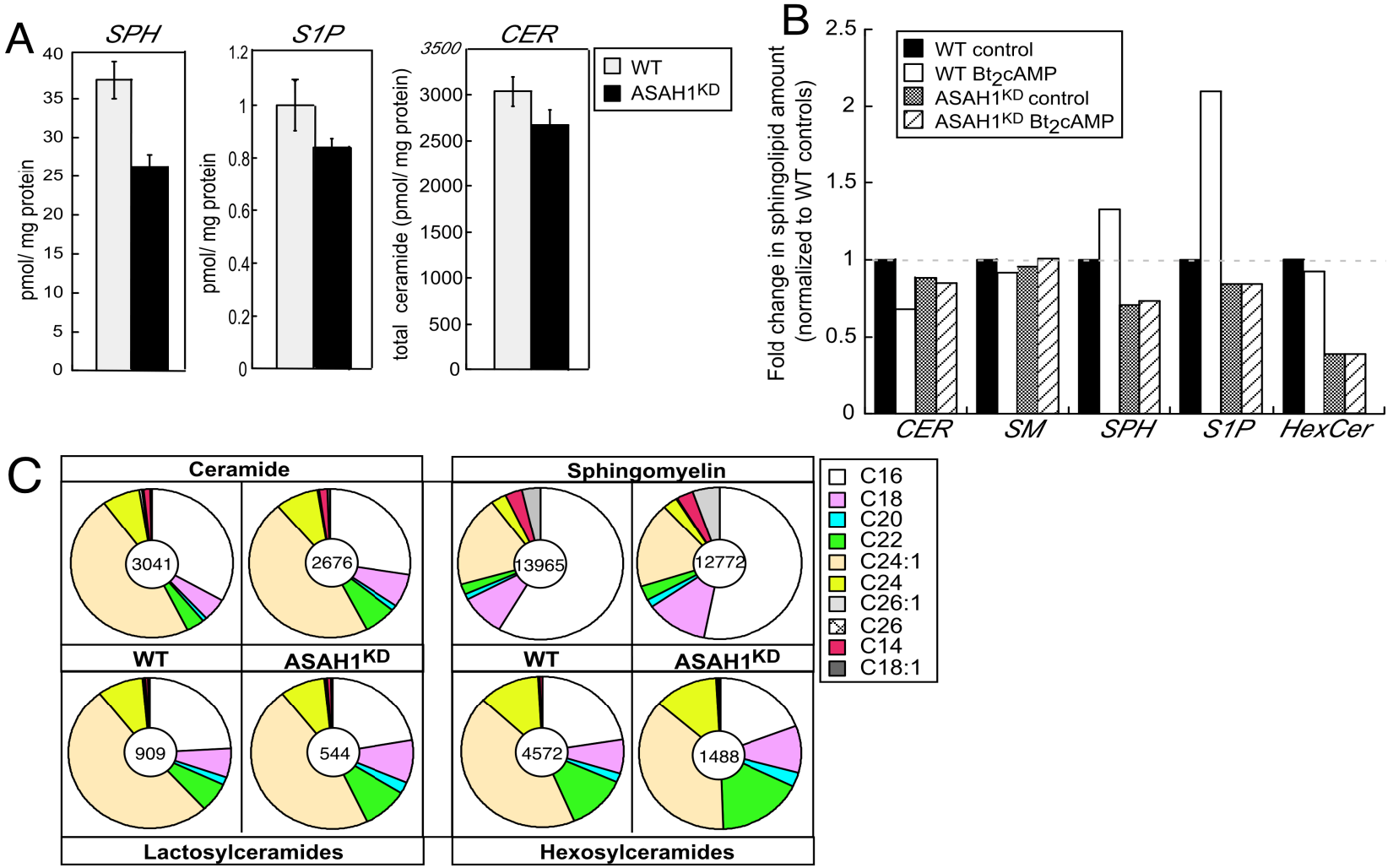



**Figure 4.5.** *ASAH1 knockdown leads to complex changes in steady-state sphingolipid content*. **A**. The intracellular amounts of sphingosine (SPH), sphingosine-1-phosphate (S1P), and ceramide (CER) were quantified in H295R wild type (WT) and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 96 h) cells by LC-ESI-MS/MS as described in Section 4.2. **B.** LC-ESI-MS/MS quantification analysis of the intracellular amounts of ceramide (CER), sphingomyelin (SM), sphingosine (SPH), sphingosine-1-phosphate (S1P), and hexosylceramide (HexCer) in H295R WT and ASAH1$^{KD}$ cells treated with 0.4 mM $Bt_2$cAMP for 30 min. Data is graphed as fold change of sphingolipid content and normalized to untreated controls. **C**. The intracellular content of ceramide, sphingomyelin, lactosylceramide, and hexosylceramide subspecies was quantified in H295R WT and ASAH1$^{KD}$ cells by LC-ESI-MS/MS. The relative distribution of different subspecies are represented with the chain length and number of double bonds depicted in the legend by x:y, respectively. Pie charts represent the relative changes in sphingolipid subspecie abundance between H295R WT and ASAH1$^{KD}$ cells. The number at the center of each chart refers to the total amount of each sphingolipid species quantified.

subspecies of LacCer and HexCer mirroring the pattern of acyl-chain composition decreases observed for SM subspecies (Figure 4.5C).

### *4.3.6. Depletion of ASAH1 alters the expression of various nuclear receptor genes*

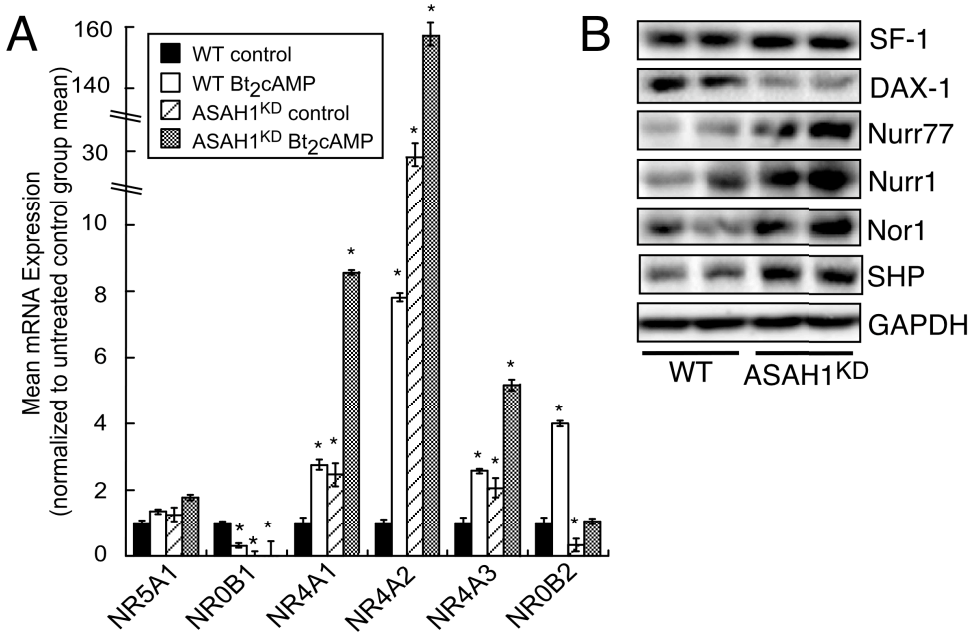



**Figure 4.6.** *ASAH1$^{KD}$ cells display altered expression of multiple steroidogenesis-associated nuclear receptor genes.* **A**. H295R wild type (WT) and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 72 h) cells were treated with 0.4 mM $Bt_2$cAMP for 18 h. Total RNA was isolated and the mRNA levels of NR5A1, NR4A1, NR4A2, NR4A3, NR0B1, and NR0B2 were quantified by qRT-PCR and normalized to the mRNA expression of β-actin. Data is graphed as mean +/- SEM of three separate experiments, each done in triplicate. Asterisks (*) indicate statistically significant differences from untreated WT cells ($p < 0.05$). **B**. H295R WT and ASAH1$^{KD}$ cell lysates were separated by SDS-PAGE and the protein expression of SF-1 (encoded by NR5A1), Dax-1 (NR0B1), Nur77 (NR4A1), Nurr1 (NR4A2), Nor-1 (NR4A3), SHP (NR0B2), and GAPDH was quantified by western blotting.

Several nuclear receptors regulate the transcription of steroidogenic genes (427). Quantitative RT-PCR analysis of genes encoding various nuclear receptors revealed that suppression of ASAH1 expression significantly increased the mRNA levels of NR4A1 and NR4A2 by 2.4- and 30.5-fold, respectively (Figure 4.6A). NR4A1 encodes NGFI-B/Nur77, which regulates the transcription of CYP21A2 (452) and StAR (439),

while NR4A2 (encodes Nurr1) activates CYP11B2 gene expression in response to angiotensin II (25). The transcription of NR4A3, which encodes the neuron-derived orphan receptor (Nor-1) and regulates CYP21A2 gene transcription (437), was induced by 2.1-fold in ASAH1$^{KD}$ cells (Figure 4.6A). Notably, ASAH1 suppression potentiated $Bt_2$cAMP-induced NR4A1, NR4A2, and NR4A3 mRNA expression. In contrast to the induction observed for NR4A family members, ASAH1$^{KD}$ cells exhibited an 89% and 66% decrease in NR0B1 (Dax-1) and NR0B2 (SHP, small heterodimer partner) mRNA transcripts, respectively (Figure 4.6A). Consistent with the changes in mRNA expression, Nur77, Nurr1 and Nor-1 protein levels were increased in ASAH1$^{KD}$ cells while Dax-1 protein expression was decreased under the same conditions (Figure 4.6B). Intriguingly, even though SHP mRNA expression was reduced in ASAH1$^{KD}$ cells (Figure 4.6A), protein expression was increased by 1.9-fold (Figure 4.6B). Finally, although SPH is a ligand for SF-1 (47), reduced levels of this sphingolipid in ASAH1$^{KD}$ cells (Figure 4.5A) did not affect the mRNA or protein expression of this receptor (Figure 4.6A and 4.6B).

### *4.3.7. ASAH1 regulates intracellular cAMP levels and ACTH responsiveness*

Because downregulation of ASAH1 altered the transcription of multiple cAMP-regulated genes (Figures 4.2 and 4.6), I hypothesized that ASAH1 suppression increased cAMP amounts. To test this hypothesis, the amount of intracellular cAMP in WT and ASAH1$^{KD}$ cells under basal and ACTH-stimulated conditions was quantified. Unexpectedly, basal intracellular cAMP levels were decreased by 46% in ASAH1$^{KD}$ cells compared to WT (Figure 4.7). However, ASAH1$^{KD}$ cells displayed a significantly higher increase in the magnitude (6.1-fold) of intracellular cAMP produced in response to ACTH when compared to WT cells (2.3-fold).

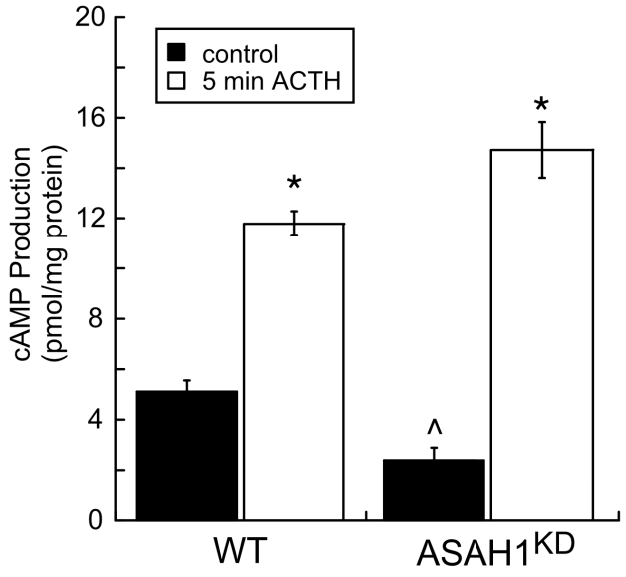



**Figure 4.7.** *ASAH1 regulates intracellular cAMP levels and ACTH responsiveness.* H295R wild type (WT) or ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 96 h) cells were treated with 50 nM ACTH for 5 min. Intracellular cAMP levels were quantified by ELISA as described in Section 4.2. cAMP amounts are normalized to total cellular protein content and data graphed represents the mean +/- SEM of three experiments, each done in triplicate. Asterisks (*) and carats (^) indicate statistically significant differences from untreated controls within each group or from untreated WT controls, respectively ($p < 0.05$).

### *4.3.8. ASAH1 knockdown affects histone H3 acetylation levels at target promoters*

Because ASAH1 suppression altered the transcriptional rate of multiple genes, I postulated that ASAH1 might alter nuclear patterns of histone acetylation. Western blotting analysis of histone H3 acetylation in WT and ASAH1$^{KD}$ cells revealed that suppression of ASAH1 resulted in a 1.7-fold increase in total acetyl-histone H3 levels (Figure 4.8A). Therefore, ChIP analysis in WT and ASAH1$^{KD}$ cells was carried out to quantify the relative amounts of acetylated histone H3 at the proximal promoter region of a subset of steroidogenic genes that exhibited altered mRNA expression in response to ASAH1 knockdown (Figures 4.2A, 4.2B, and 4.6A). Consistent with increased mRNA expression, acetyl-histone H3 levels at the proximal promoter region of StAR, Mc2R, NR4A2, and CYP17A1 genes were increased by 4.2-, 21.1-, 3.2-, and 4.1-fold respectively, in ASAH1$^{KD}$ cells compared to WT (Figure 4.8B). Further, a 8.6-fold decrease in histone H3 acetylation at the DAX-1 proximal promoter was observed in ASAH1$^{KD}$ cells (Figure 4.8B).

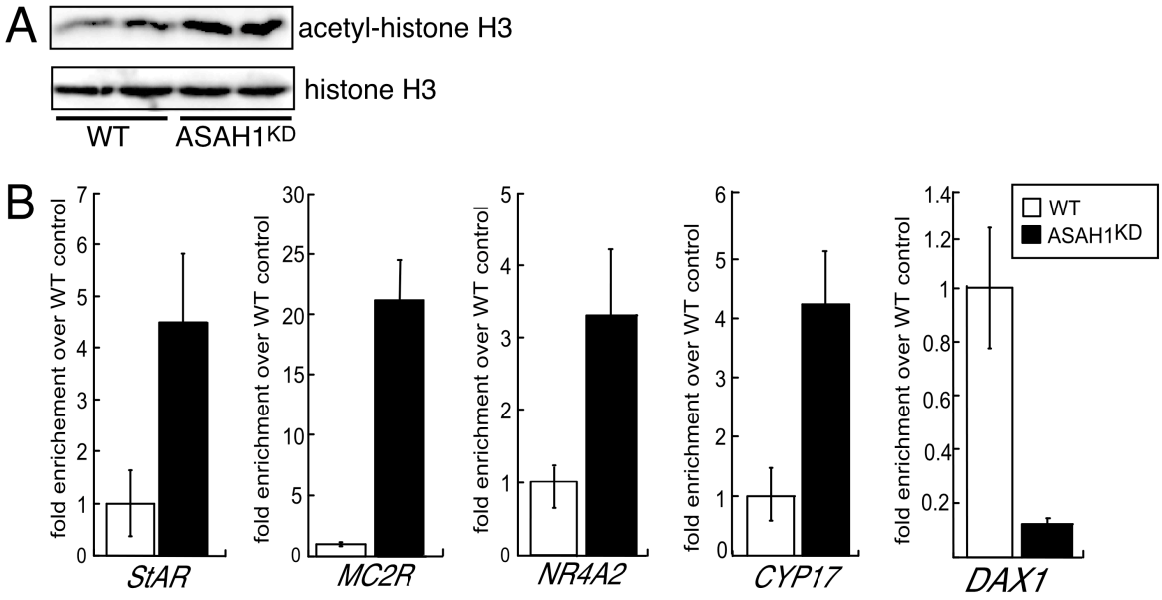



**Figure 4.8.** *Suppression of ASAH1 affects histone H3 acetylation levels.* **A.** H295R wild type (WT) and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 96 h) cell lysates were isolated and separated by SDS-PAGE. Acetyl-histone H3 and total histone H3 levels were determined by western blotting. **B.** Graphical analysis of data obtained from western blotting studies of acetyl-histone H3 protein expression in WT and ASAH1$^{KD}$ cells. Data graphed represents the mean +/- STDEV of 3 separate experiments, each done in duplicate. H295R WT and ASAH1$^{KD}$ (pre-treated with 5 μg/mL tet for 96 h) cells were crossed linked with 1% formaldehyde and the sheared chromatin immunoprecipitated with an anti-acetyl histone H3 antibody. Acetyl-histone H3 levels at the proximal promoter regions of StAR, Mc2R, CYP17A1, NR4A2, and DAX-1 genes were assessed by qPCR. Purified DNA was quantified by real time PCR and normalized to the ΔCt values of input DNA. Data is expressed as fold change over untreated WT controls.

## 4.4. Discussion

In the zonae fasciculata and reticularis of the human adrenal cortex, steroid hormone biosynthesis requires the coordinate action of steroid hydroxylases and dehydrogenases (126,426,453), lipoprotein receptors (448,454), and cholesterol binding proteins (17,455-458). The transcription of most of these genes is regulated by ACTH, which upon binding to Mc2R activates a cAMP-signaling cascade. Sphingolipids, such as Cer, SPH, and S1P, regulate steroidogenesis by acting as secondary modulators of steroidogenic gene transcription (47,115,205-206,210) and/or steroidogenic regulatory pathways (175-176,202,204,207,212,396,459-460). Previous work from our group has demonstrated that dynamic sphingolipid metabolism is a component of ACTH/cAMP-dependent steroidogenesis (75,461).

A hallmark of adrenocortical steroid hormone biosynthesis is the induction of steroidogenic gene transcription (Figure 4.2A) in response to chronic ACTH stimulation. The studies presented in this Chapter demonstrate that ASAH1 suppression mimics ACTH/cAMP-dependent CYP11A1, CYP17A1, CYP11B, and CYP21A2 gene expression and potentiates the effect of $Bt_2$cAMP on CYP11A1 and CYP21A2 mRNA levels (Figure 4.2B). Moreover, depletion of ASAH1 resulted in the upregulation of StAR, TSPO, HSL, and SR-BI transcription (Figure 4.2C), all of which encode proteins that are required for the cellular uptake of lipoproteins, the de-esterification of cholesteryl esters, and the transport of free cholesterol into the inner mitochondria membrane (7,12,16,462). The effect of ASAH1 suppression on the transcription of these steroidogenic genes is consistent with previously published data from our laboratory establishing SPH as an antagonist of SF-1 and suppressor of CYP17A1 transcription (298). Because SF-1 regulates the transcription of CYP17A1, CYP11A1, StAR, Mc2R, and CYP11B1 (264,432), lower SPH intracellular levels (Figure 4.5A) as a result of ASAH1 knockdown positively correlates with increased basal mRNA expression of these genes (Figure

4.4B). Altered steroidogenic gene expression in ASAH1$^{KD}$ cells can also be explained by higher mRNA and protein levels of the nuclear receptors Nur77 and Nor-1 (Figures 4.7A and 4.6B), both of which regulate CYP21A2 transcription (437,452). Nur77 also modulates StAR gene expression (439). Further, the expression of the transcription factor Dax-1 is significantly downregulated in ASAH1$^{KD}$ cells (Figures 4.6A and 4.6B). Dax-1 represses the transactivation potential of many nuclear receptors (463), including SF-1 (436,464), and its expression negatively regulates steroid hormone production at multiple levels (463,465-467). Furthermore, the absence of Dax-1 potentiates the ability of adrenal cells to respond to ACTH *in vivo* (435). Therefore, repression of this nuclear receptor may contribute to increased steroidogenic gene expression in ASAH1$^{KD}$ cells. The fact that not all SF-1-regulated genes are basally upregulated in ASAH1$^{KD}$ cells (e.g. 3β-HSD) suggests that additional factors are required for inducing the expression of those genes. Consistent with mRNA and protein expression data (Figure 4.2), ASAH1$^{KD}$ cells secrete significantly higher levels of cortisol and DHEA than WT cells, both basally and in response to $Bt_2$cAMP stimulation (Figure 4.3). ASAH1 knockdown also increased the magnitude of cAMP production in response to ACTH (Figure 4.7). This finding positively correlates with higher mRNA and protein expression of Mc2R (Figures 4.2C and 4.2D), the gene that encodes the cognate receptor for ACTH.

Significantly, ASAH1 suppression altered the amount of acetylated histone H3 at the proximal promoter region of multiple genes, including Mc2R, StAR, DAX-1, CYP17A1, and NR4A2 (Figure 4.8B). Lysine acetylation of histones is an epigenetic modification that is usually associated with active chromatin regions (468). Conversely, decreased mRNA and protein levels of DAX-1 in ASAH1$^{KD}$ cells (Figure 4.6) positively correlated with lowered amounts of acetylated histone H3 (Figure 4.8B). Sphingolipids are rapidly emerging as important mediators of various nuclear processes (444). Recently, Hait *et al.* (255) uncovered a novel role for nuclear SPHK2 and S1P in

regulating HDAC activity and histone acetylation. S1P was found to inhibit HDAC1 and HDAC2 enzymatic activity and enhance local histone H3 acetylation and transcription of target genes (255). Along this line of evidence, it would follow that lower ASAH1 expression would result in lower S1P levels and thus decreased histone H3 acetylation. However, my data show that ASAH1 suppression leads to increased cellular amounts of acetylated histone H3 (Figure 4.8A) and an increase in the acetylation of histone H3 levels at selected promoters (Figure 4.8B). Given that I observed no significant change in S1P levels in ASAH1$^{KD}$ cells (Figure 4.5A), this discrepancy between my findings and the work of Hait *et al.* may reflect cell-specific differences. Of note, intracellular S1P amounts remained unchanged in response to ASAH1 suppression despite an increase in SPHK1 and SPHK2 protein levels (Figure 4.4D), which is likely due to lower substrate (SPH) amount (Figure 4.5A).

ASAH1 knockdown had various effects on the transcription of sphingolipid genes (Figure 4.4). However, the changes observed in mRNA expression cannot fully explain the changes seen in the steady-state pool of sphingolipids (Figure 4.5). Total Cer and SM levels were unchanged in ASAH1$^{KD}$ cells, while the total amounts of LacCer and HexCer were significantly lower compared to WT cells (Figure 4.5B). As predicted, a reduction in SPH levels in ASAH1$^{KD}$ cells was observed (Figure 4.5A), however, this decrease was inconsistent with the lack of a significant change in total Cer levels. Given that ASAH1 catalyzes Cer hydrolysis and Cer degradation is the only source of cellular SPH (104), I anticipated that Cer would accumulate in these cells. Meroni *et al.* (205) previously reported that inhibition of ceramidase activity by (1S,2R)-d-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-e-MAPP), an inhibitor of ACER3 (469), resulted in Cer accumulation and led to a decrease in testosterone secretion from rat gonadal Leydig cells. Although the cell system used in the current study differs from the one used by Meroni *et al*., the quantitative differences in Cer amounts when comparing these two

studies support the notion that each ceramidase isoform has unique cellular functions. This premise is supported by the differences in subcellular localization (94-99) and substrate specificity (96,100-103) of each ceramidase isoform.

The data presented in this Chapter indicate that ASAH1 knockdown had varied effect on sphingolipid homeostasis. Depletion of ASAH1 caused a shift in the acyl-chain lengths of Cer, SM, HexCer, and LacCer (Figure 4.5B). There was an accumulation in saturated long-chain ($C_{18-24}$) Cer and SM species, a decrease in unsaturated $C_{24:1}$- and $C_{26:1}$-Cer, and an overall reduction in $C_{16}$-sphingolipids. A decrease in unsaturated very long-chain Cer species may be partially due to decreased mRNA levels of Elovl2 (Figure 4.4C), which catalyzes the elongation of polyunsaturated fatty acyl-CoA of up to 24 carbons (450), and lower CerS2 protein levels (Figure 4.4D), which uses very long-chain acyl-CoAs as substrates (449). It is equally probable that the protein levels and/or activity of fatty acyl-CoA elongases as well as the enzymatic activity of different CerS enzymes are increased in ASAH1$^{KD}$ cells and may contribute to the acyl-chain composition of sphingolipids in these cells. Interestingly, the levels of $C_{16}$-Cer species are decreased in ASAH1$^{KD}$ cells despite an increase in CerS6 protein levels (Figure 4.4D). Also, although ACER3 was recently reported to hydrolyze unsaturated long-chain $C_{18:1}$-Cer (100), higher levels of this particular Cer subspecies in ASAH1$^{KD}$ cells were observed, which was inversely correlated to the levels of ACER3 mRNA expression in these cells (Figure 4.4A). The underlying mechanism for these changes in the sphingolipidome is unknown and further studies are necessary to elucidate the specific roles of ASAH1 in regulating sphingolipid acyl-chain composition. However, it is notable that similar unpredicted changes in sphingolipid content were recently observed by Mullen *et al.* (470) in response to CerS2 knockdown. Conversely, Park *et al.* (471) reported that the lipid backbone composition of Cer subspecies and fatty acyl-CoAs from mouse embryonic stem cells and embryoid bodies correlated fairly well with CerS and

Elovl genomic analysis. Thus, the changes in sphingolipid amounts resulting from sphingolipid gene knockdown are multifaceted and sometimes cannot be readily explained by changes in gene expression. Given that recently lipidomic analysis has revealed that Cer with varying fatty acyl-chain length may have distinct cellular functions (61-62), it is tempting to speculate that some of the physiological changes observed in response to ASAH1 suppression are partially due to a shift in the acyl-chain length of sphingolipid species.

Because ceramidases regulate the intracellular levels of two proliferation-associated molecules, they have key implications in cell fate. ASAH1 is overexpressed in various cancers (110-113) while ASAH1 knockout leads to embryonic lethality in mice (109). Further, ACER3 and ASAH2 were both reported to modulate cell proliferation either by upregulating cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ expression (100) or inducing cell cycle arrest at $G_0/G_1$ and retinoblastoma (Rb) protein dephosphorylation (472), respectively. Accordingly, the results presented in this Chapter suggest that ASAH1 controls the rate of cell proliferation in H295R cells (Figure 4.3B), at least in part, by decreasing the expression of multiple proliferation protein markers including β-catenin, PCNA, and cyclin B2 (Figure 4.3C). Because ASAH1 suppression had no effect on cell viability (data not shown), apoptosis was excluded as a reason for reduced proliferation. The present study is consistent with my findings in MCF-7 breast cancer cells where I observed that suppression of ASAH1 expression by siRNA prevented genistein-induced cyclin B2 protein expression (Appendix 1) and further supports a role for ASAH1 in controlling cell growth, at least in part, through the regulation of proliferation-associated protein expression (Figure 4.3C). Additionally, emerging evidence suggest that LacCer levels positively correlate with various phenotypic changes including cell proliferation and migration (473-475). Therefore, lower LacCer

amounts in response to ASAH1 knockdown may also contribute to the decrease in proliferation of ASAH1$^{KD}$ cells.

In summary, this Chapter describes that ASAH1 knockdown leads to global changes in various classes of genes, including steroidogenic, nuclear receptors, and sphingolipid-metabolizing enzymes. ASAH1$^{KD}$ cells secrete significantly higher levels of cortisol and DHEA than WT cells, which positively correlate with higher mRNA expression of CYP11A1, CYP11B, CYP17A1, CYP21A2, StAR, TSPO, HSL, SR-BI, and Mc2R genes. These changes were largely the result of increased acetylation of histone H3 at the proximal promoters of these genes. ASAH1$^{KD}$ cells were also more sensitive to ACTH and displayed 6-fold higher intracellular cAMP accumulation upon hormone stimulation than WT cells, likely due to induced Mc2R expression (Figure 4.2D). Intriguingly, ASAH1 silencing resulted in the induction of all members of NR4A subfamily of nuclear receptors and the suppression of DAX-1 (Figure 4.6), indicating a novel role for this ceramidase in modulating nuclear receptor expression. Finally, I demonstrate that downregulation of ASAH1 causes a shift in sphingolipid metabolism towards Cer, SM, LacCer, and HexCer subspecies with long saturated fatty acyl-chain lengths. I conclude that ASAH1 is a global mediator of steroidogenic gene expression and adrenocortical steroidogenesis.

# CHAPTER 5:

*Acid ceramidase represses steroidogenic factor-1-dependent gene transcription in H295R human adrenocortical cells by binding to the receptor*

## 5.1. Introduction

ACTH regulates cortisol biosynthesis in the human adrenal cortex by activating a cAMP/PKA-dependent signaling pathway that leads to rapid cholesterol import and transport, as well as the transcriptional activation of genes required for steroid hormone production (27,31). The transcription of most steroidogenic genes is regulated by SF-1 (NR5A1), which in response to ACTH signaling binds to target promoters and facilitates the recruitment of coactivator proteins (27,134,149,156,476). SF-1 is a member of the nuclear receptor superfamily of transcription factors (117) whose structure is divided into modular motifs: an amino-terminal conserved DNA binding domain (DBD) consisting of two zinc-binding modules, an intervening hinge region containing a ligand-independent activation domain (AF1), and a carboxy-terminal ligand-binding domain (LBD) containing a conserved AF-2 hexamer domain (LLIEML) that is critical for receptor activation (118) (Figure 1.5). The hinge region and LBD participate in transcriptional repression or ligand-dependent activation. These domains serve as the interface for interactions between SF-1 and numerous coregulatory proteins, including SRC-1 (119-120) and SMRT (121). Generally, coregulators bind to the AF1 and/or AF2 domains of nuclear receptors through LXXLL motifs (NR boxes), where X is any amino acid and L is a leucine (477-478). Additional LXXLL-related motifs where L is substituted for an isoleucine, phenylalanine, or methionine have also been reported (169-170).

The ability of SF-1 to bind to target promoters is regulated by post-translational modifications including phosphorylation (148-151), sumoylation (152-153), and acetylation (155-156,476), as well as protein-protein interactions (126,157-162). More recently, ligand binding has also been implicated in the regulation of SF-1 activity

(47,163-165,167). Crystallographic studies using bacterially expressed SF-1 have demonstrated that phospholipids are present in the ligand-binding pocket and that ligand binding is required for maximal activity of the receptor (163,165,167,244). Our laboratory has recently identified PA and SPH as endogenous ligands for SF-1 (47,164). PA acts as an agonist while SPH is an antagonist for the receptor. SPH is bound to SF-1 under basal conditions and prevents receptor binding to the CYP17A1 promoter, thus decreasing $Bt_2$cAMP-stimulated CYP17A1 mRNA expression and DHEA synthesis (47). Further, as discussed in Chapter Four, I have uncovered a global role for ASAH1, an enzyme that produces SPH, in steroidogenic gene transcription in adrenocortical cells. ASAH1 knockdown induces the expression of multiple SF-1-target genes including StAR, Mc2R, and CYP17A1, and results in increased cortisol and DHEA secretion (Figure 4.3), which supports a role for ASAH1 in regulating the transactivation potential of SF-1.

Because Cer degradation is the only source of cellular SPH (104), ASAH1 is not only essential for limiting Cer-induced signaling but also controlling the cellular functions of SPH and the counter-regulatory functions of Cer and S1P (83,105-106). Multiple factors regulate ceramidase activity including dexamethasone (72,479), PMA (480), and TNF-$\alpha$ (67,71,80). Additionally, *in vivo* studies have shown that ASAH1 requires sphingolipid activator proteins (SAP, saposin), mainly SAP-D, as cofactors for maximal activity (481). ASAH1 has been reported to localize to lysosomes (99) and to be secreted extracellularly from murine endothelial cells, macrophages, and human fibroblasts (98). This ceramidase is required for developmental because targeted disruption of the gene in mice leads to an early, embryonic lethal phenotype (109). In addition, a genetic deficiency in ASAH1 resulting in reduced enzymatic activity causes Farber's disease, a lysosomal sphingolipid storage disorder (114).

Based on previous findings from our group establishing SPH as an antagonist for SF-1 (47) and that SPH-mediated repression of CYP17A1 transcription is dependent on ASAH1 expression (47), as well as the role of ASAH1 in global steroidogenic gene expression and steroid hormone secretion (Chapter Four), the aim of the current study was to define the mechanism by which ASAH1 regulates SF-1 function in adrenocortical cells. In this Chapter I show that ASAH1 and its coactivator protein SAP-D are localized in the nuclei of H295R cells and that ACTH/cAMP signaling acutely increases ASAH1 activity, concomitant with the nuclear metabolism of Cer and SPH. Significantly, ASAH1 represses SF-1 activity by directly binding to the receptor through a functional LXXLL-related motif. This interaction occurs on DNA and is strengthened by $Bt_2$cAMP stimulation. Furthermore, the catalytic activity of ASAH1 is required for its ability to repress SF-1 function. Finally, I show that ASAH1 is recruited to the promoter of various SF-1-target genes, suggesting a global coregulatory role for ASAH1 in adrenocortical cells.

## 5.2. Materials and methods

### *5.2.1. Cell culture*

H295R adrenocortical cells and H295R ASAH1$^{KD}$ (ASAH1-knockdown) cells were cultured as described in Chapters Two and Four, respectively.

### *5.2.2. Nuclear extract purification and western blotting*

H295R cells were sub-cultured into 100-mm dishes and cytoplasmic, nuclear envelope, and nucleoplasmic fractions were purified using the Nuclei Pure Isolation Kit (Sigma) following the manufacturer's instructions. Aliquots (30 μg of protein) of cytoplasmic, nuclear envelope, and nucleoplasmic fractions were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Temecula, CA). Blots were probed with antibodies against SF-1 (Millipore), ASAH1 (HPA005468, Sigma), ASAH2 (PRS4743, Sigma), ACER3 (sc-101848, Santa Cruz, St. Cruz, CA), saposin D (sc-27024-R, Santa Cruz), lysosomal-associated membrane protein 1 (LAMP1) (sc-18822, Santa Cruz), P450c17α (sc-66849, Santa Cruz), or StAR (sc-25806, Santa Cruz). Expression was detected using an ECF Western blotting kit (Amersham Bioscience, Piscataway, NJ) and visualized by scanning blots on a Versa Doc 4000 imager (Bio Rad, Hercules, CA)

### *5.2.3. Immunostaining and confocal microscopy*

Immunostaining and confocal microsopy were performed as described previously (164). Briefly, H295R cells were plated on glass cover slips, fixed, permeabilized, blocked with 1% BSA for 1 h, and then incubated with anti-ASAH1, anti-ASAH2, anti-ACER3, or anti-saposin D antibodies (diluted 1:200 in 1% BSA) overnight, followed by incubation with secondary goat anti-rabbit rhodamine-conjugated IgG (Thermo Scientific) for 1 h. Cover slips were stained with 4,6'-Diamino-2-phenylindole

dihydrochloride (DAPI; Invitrogen) for 2 min and then mounted onto slides using Fluoromount G (Southern Biotech, Birmingham, AL). Confocal images were captured using a laser-scanning microscope (LSM 510; Zeiss, Thornwood, NY) equipped with argon and helium-neon laser with excitation wavelengths of 436 and 542 nm for DAPI and rhodamine, respectively.

### *5.2.4. In situ ceramidase activity assay*

H295R wild type (WT) or ASAH1$^{KD}$ [pre-treated for 96 h with 5 μg/mL tetracycline (tet)] cells were sub-cultured into 100-mm dishes, incubated for 4 h with 4 μM NBD-12-Cer {N-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl-amino) dodecanoyl]-D-*erythro*-sphingosine}, and then treated for 30 min to 6 h with 0.4 mM $Bt_2$cAMP. After treatment, cells were harvested into lysis buffer [0.2% Triton X-100, 10 mM Tris-Cl pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA, 15 mM NaCl, 1X protease inhibitor cocktail set I (EMD Biosciences)] and sonicated 5 times for 2 sec. Total lipids were extracted by adding 2.5 mL chloroform/methanol (2/1, v/v), sonicating 5 times for 15 sec, and vortexing for 2 min. Samples were centrifuged (10 min at 4,000 rpm) and the organic phase dried under a stream of $N_2$. Lipids were spotted on Silica Gel 60 thin-layer chromatography (TLC) plates (EMD chemicals Inc., Gibbstown, NJ). Plates were developed in chloroform:methanol:25% ammonium hydroxide (90:20:0.5, v/v/v) and visualized by fluorescence scanning on a VersaDoc 4000 imager (Bio-Rad). NBD-dodecanoic acid {12-[N-(7-nitro-2-1,3-benzoxadiazol-4-yl-amino) amino]-dodecanoic acid} formation was quantified and normalized to the protein content of each sample.

### *5.2.5. Analysis of sphingolipid species*

Sphingolipid concentrations in H295R WT and ASAH1$^{KD}$ (pre-treated for 96 h with 5 μg/mL tet) nuclei were analyzed by liquid chromatography, electrospray ionization,

tandem mass spectrometry (LC-ESI-MS/MS) as described previously (446-447). Nuclei were purified using the Nuclei PURE Prep kit (Sigma) following the manufacturer's instructions.

### *5.2.6. Transient transfection and reporter gene analysis*

H295R cells were transfected with 100 ng pGL3-CYP17A1-2x57 reporter plasmid (126), 25 ng pCMV6myc/FLAG-SF1 (RC207577, Origene, Rockville, MD), and/or 50 ng pCMV6myc/FLAG-ASAH1-wild type (WT), pCMV6myc/FLAG-ASAH1-LXXLL1, pCMV6myc/FLAG-ASAH1-LXXL5, 25 ng pBKCMV-SRC-1 or pCMX-SMRT using GeneJuice (EMD Biosciences). Cells were co-transfected with 1.5 ng of a Renilla luciferase plasmid under the control of the thymidine kinase (TK) promoter (pRL-TK, Promega, Madison, WI). Twenty-four h after transfection, cells were treated with 0.4 mM $Bt_2$cAMP for 16 h. In some experiments, cells were pre-treated for 1 h with 0.5 μM or 5 μM D-NMAPPD {N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)-ethyl]-tetradecanamide} before treatment with $Bt_2$cAMP. Firefly and Renilla luciferase activities were determined using a dual luciferase assay (Promega) and CYP17A1 reporter gene activity (pGL3, Firefly) was normalized to Renilla activity.

### *5.2.7. Coimmunoprecipitation (coIP)*

For endogenous protein interaction assays, H295R cells were treated with 0.4 mM $Bt_2$cAMP for 30 min. coIP assays were also performed with tagged proteins by transfecting CV-1 monkey kidney cells with 5 μg of pCMV6-GFP-hSF-1 (RG207577, Origene) and pCMV6myc/FLAG-ASAH1 wild type (WT) or pCMV6myc/FLAG-ASAH1-LXXLL1, pCMV6myc/FLAG-ASAH1-LXXLL5 mutant expression plasmids for 72 h. Cells were washed twice with PBS and harvested into RIPA buffer containing protease inhibitors (EMD Chemicals). Lysates were then sonicated 6 times for 5 sec followed by

centrifugation at 12,000 rpm for 15 min at 4°C. Samples were pre-cleared with 20 μL BSA (20 mg/mL) and immunoprecipitated overnight at 4°C on a tube rotator using protein A/G Plus agarose (Santa Cruz) and anti-SF1 (Millipore, for H295R lysates) or anti-FLAG (Sigma, for CV-1 lysates) antibodies. The immobilized protein complexes were washed twice in RIPA buffer and twice in PBS, and then separated by SDS-PAGE. Western blots were probed with anti-ASAH1, anti-saposin D, anti-SF1, or anti-GFP (Invitrogen) antibodies and expression was detected using an ECF Western Blotting Kit (GE Biosciences) and visualized using a VersaDoc 4000 imager (Bio-Rad).

### *5.2.8. In vitro binding assays*

His-tagged SF-1 was expressed and purified from E. coli as previously described (482). ASAH1 was *in vitro* translated from the pCMV6-XL5-ASAH1 plasmid (RC212434, Origene) using the TNT Quick Coupled Transcription/Translation Systems kit (Promega). ASAH1 expression was confirmed by subjecting samples to SDS-PAGE, coomassie staining, and exposing the gel to a radioactive-imager screen. ASAH1 expression was visualized by radioactive-imager scanning (FLA 7000, Fuji, Japan). Twenty μL of *in vitro* translated ASAH1 was incubated with 50 μL His-tagged immobilized SF-1, 10 μL BSA (10 mg/mL), and 1 mL buffer (4 M NaCl, 160 nM Tris-HCl, 40 mM imidazole, pH 7.9) in a tube rotator for 24 h at 4°C. Complexes were washed 3 times and subjected to SDS-PAGE and coomassie staining. Gel was dried, exposed to a phosphor-imager screen, and visualized by phosphor-imager scanning (Fuji, Japan).

### *5.2.9. Site-directed mutagenesis*

Mutagenesis was carried out using a Stratagene QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. To mutate

putative nuclear receptor binding (LXXLL) motifs in ASAH1, the first and fourth leucines within each motif were mutated to alanine using the following primers: ASAH1-LXXLL1 (5'- TAC CCA AGT GCC AGC GCG GCT TTC ACC GAG -3'), ASAH1-LXXLL5 (5'-GTG GGC ATG GCT ACA GGA GCT AAA CCA GGA-3'). To generate truncation mutants of ASAH1 [$\Delta\alpha$subunit ($\Delta\alpha$) or $\Delta\beta$subunit ($\Delta\beta$)] the following primer sets were used: pACT-ASAH1$\Delta\alpha$ (forward 5'-GGG ATC CGT CGA CTT ATG AAC TGC TGC ATC GGG-3'; and reverse 5'-TCT AGA TGA TAT CAA AAT GGT AAA TAATTC ATA-3') and pACT-ASAH1$\Delta\beta$ (forward 5'-GGG ATC CGT CGA CTT TGT ACT TCA ATA GTA GCA-3'; and reverse 5'-TCT AGA TGA TAT CAA CCA ACC TAT ACA AGG GTC-3').

### *5.2.10. Mammalian 2-hybrid assay*

ASAH1 (full-length and truncation mutants) and SF-1 genes were cloned into the *SalI* and *EcoRV* sites of pBIND and/or pACT vectors (Promega). H295R cells were sub-cultured into 24-well plates and transfected with the pG5 firefly luciferase reporter in combination with pBIND and pACT vectors expressing fusions of Gal4 DNA binding domain and VP16 activation domain, respectively, with ASAH1 (wild type, $\Delta\alpha$, or $\Delta\beta$) or SF-1. In some experiments, cells were co-transfected with 50 ng of SRC-1 or SMRT expression plasmids. The ratio of pG5 to pBIND to pACT in transient transfections was 50 ng: 50 ng: 50 ng. Cells were incubated for 48 h before harvesting and assaying for dual luciferase activity.

### *5.2.11. Chromatin immunoprecipitation (ChIP)*

ChIP assay was performed as previously described (158). Briefly, H295R cells were sub-cultured into 150-mm dishes and then treated with 0.4 mM $Bt_2$cAMP for 30 min. After cross-linking with 1% formaldehyde, cells were harvested into RIPA buffer and

lysates sonicated to obtain optimal DNA fragments lengths of 100-1000 bp (5 times for 10 sec) followed by centrifugation at 12,000 rpm for 15 min at 4°C. The purified chromatin was pre-cleared with 1 μg IgG and immunoprecipitated overnight at 4°C on a tube rotator using 5 μg of primary antibody [anti-trimethyl(Lys20) histone H4 (Millipore), anti-RNA polymerase II CTD (Millipore), anti-SF-1 (07-618, Millipore), anti-ASAH1 (HPA005468, Sigma), anti-SRC-1 (Millipore), and anti-HDAC1 (Millipore)] and 30 μL protein A/G Plus agarose (Santa Cruz). Real Time PCR was carried out using 20% of output, 5% input (diluted 1:4), the ABsolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific), and the following primer sets: forward, 5'-GGC TGG GCT CCA GGA GAA TCT TTC TTC CAC-3'; reverse, 5'-CGG CAG GCA AGA TAG ACA GCA GTG GAG TAG-3', which amplify the region of the CYP17A1 promoter from position -104 to +43 that encompass an essential SF-1 binding site. For determination of ASAH1 enrichment at other gene promoters, the following primers were used: Mc2R (forward 5'-TTG CCC AGA AAG TTC CTG CTT-3', and reverse 5'-TTC TCC TGC TTG TGG TTA AGG-3'), and DAX-1 (forward, 5'-TCC AGC TTT TAA AGA GCA CCC GCC CCT-3'; and reverse 5'-CGG CGC CCG TAG CCC AGT TCT-3'), which amplify the proximal (-300 bp) promoter region of each gene. For negative controls, primers for β-actin (forward, 5'-TGC ACT GTG CGG CGA AGC-3'; and reverse, 5'-TCG AGC CAT AAA AGG CAA-3') were used. PCR reactions were as follows: 1) 1 X 95°C, 15 min, 2) 60 X 95°C, 15 sec, 55°C, 30 sec, 72°C, 30 sec, 3) 1 X 95°C, 30 sec, 60°C, 30 sec, 4) 80 X 60°C, 10 sec, 5) cool to 4°C. Output ΔCt values were normalized to input values. Some PCR reactions were resolved by 2% agarose gel electrophoresis.

### *5.2.12. Sequential ChIP (re-ChIP)*

H295R cells were sub-cultured into 150-mm dishes and treated with 0.4 mM

$Bt_2$cAMP for 30 min. Cross-linking was performed by the addition of formaldehyde (final concentration of 1%) for 10 min with gentle shaking. The reaction was stopped by the addition of glycine (0.125 M final concentration) for 5 min, after which the cells were washed twice in PBS and harvested into RIPA buffer. Lysates were then sonicated to obtain optimal DNA fragments lengths of 100-1000 bp (5 times for 10 sec) followed by centrifugation at 12,000 rpm for 15 min at 4°C. Supernatant (50 μL) was retained as input. The purified chromatin was pre-cleared with 1 μg IgG and immunoprecipitated overnight at 4°C on a tube rotator using 5 μg of primary antibody [anti-SF-1 (07-618, Millipore) or anti-ASAH1 (HPA005468, Sigma)] and 30 μL protein A/G Plus agarose (Santa Cruz). The immobilized protein/DNA complexes were subjected to a series of 5-min washes: three times in RIPA buffer, three times in RIPA buffer plus 500 mM NaCl, three times in washing buffer (10 mM Tris-Cl, pH 8.0; 0.25 M LiCl; 1 mM EDTA; 1mM EGTA; 1% Nonidet P-40; 1% sodium deoxycholate), and three times in Tris-EDTA (TE) buffer, pH 8.0. Protein/DNA complexes were released from agarose beads by the addition of 50 μL TE buffer containing 10 mM dithiothreitol (DTT) and incubation at 37°C for 30 min followed by centrifugation at 4,000 rpm for 5 min. Supernatants were transferred to new tubes and 1 mL RIPA buffer was added. Chromatin was then submitted to another round of immunoprecipitation using 5 μg of primary antibody [anti-SF-1 (07-618, Millipore) or anti-ASAH1 (HPA005468, Sigma)] and 30 μL protein A/G Plus agarose (Santa Cruz) overnight at 4°C on a tube rotator. The immobilized protein/DNA complexes were subjected to a series of 5-min washes as described above. The crosslinks were reversed and protein digested using proteinase K (100 μg/mL). DNA was purified by phenol-chloroform extraction and ethanol precipitation. Real Time PCR was carried out using 20% of output, 5% input (diluted 1:4), the ABsolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific), and the following primer sets: CYP17A1

(forward, 5'-GGC TGG GCT CCA GGA GAA TCT TTC TTC CAC-3'; reverse, 5'-CGG CAG GCA AGA TAG ACA GCA GTG GAG TAG-3') or StAR (forward 5'-GCA GTG TGA GGC AAT CGC TCT-3', and reverse 5'-TGT TTC CTG GCA AAT GTG GCA-3'). For negative controls, primers for β-actin (forward, 5'-TGC ACT GTG CGG CGA AGC-3'; and reverse, 5'-TCG AGC CAT AAA AGG CAA-3') were used. PCR reactions were as follows: 1) 1 X 95°C, 15 min, 2) 40 X 95°C, 15 sec, 52°C, 30 sec, 72°C, 30 sec, 3) 1 X 95°C, 30 sec, 60°C, 30 sec, 4) 80 X 60°C, 10 sec, 5) cool to 4°C. Output ΔCt values were normalized to input values. Some PCR reactions were resolved by 2% agarose gel electrophoresis.

### *5.2.13. Statistical analysis*

One-way analysis of variance and Tukey's multiple comparison tests were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Significant differences from a compared value were defined as $p < 0.05$ and denoted by asterisks (*), carats (^), and pound (#).

## 5.3. Results

### *5.3.1. ASAH1 and SAP-D are expressed in the nucleus of H295R adrenocortical cells*

Our group has previously shown that SPH is an antagonist for SF-1 and represses the transcription of CYP17A1 (47). Moreover, as described in Chapter Four, ASAH1 silencing confers increased steroidogenic gene expression and hormone secretion. However, the mechanism by which SPH production is controlled and the parameters governing ligand binding are unknown. Because ceramidase activity is the only source of SPH (42,104) and SF-1 is a nuclear protein, confocal microscopy and western blotting were used to determine the subcellular localization of ceramidases in H295R cells. As shown in Figure 5.1A, ASAH1 and ASAH2 are expressed in the nuclear envelope and nucleoplasm while ACER3 is cytoplasmic. Furthermore, the ASAH1 coactivator protein SAP-D (483-484) is expressed in the same subcellular fractions as ASAH1 (Figure 5.1A). Because ASAH1 has optimal *in vitro* activity in acidic pH, it was

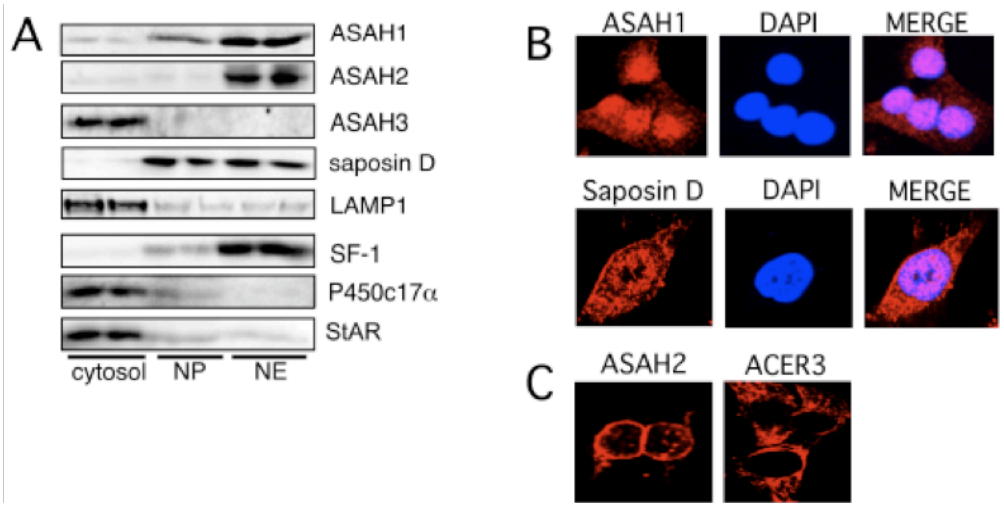



**Figure 5.1.** *ASAH1 and SAP-D are expressed in the nucleus of H295R cells*. **A.** H295R cells were sub-cultured into 100-mm dishes for 72h and cytosol, nuclear envelope (NE), and nucleoplasmic (NP) fractions were isolated as described in the Section 5.2. Lysates (30 μg of protein) were separated by SDS-PAGE followed by western blotting analysis using ASAH1, ASAH2, ACER3, SF-1, saposin D, LAMP1, P450c17α, or StAR antibodies. **B** and **C.** H295R cells were plated onto glass coverslips, fixed, permeabilized, and incubated with anti-ASAH1, anti-saposin D, anti-ASAH2, or anti-ACER3 antibodies overnight followed by a 2-min incubation with the nuclear marker DAPI. Coverslips were washed and incubated with antirhodamine and mounted, and immunofluorescence was detected by confocal microscopy.

classified as a lysosomal protein (99). Therefore, to confirm the purity of the nuclear extracts, I determined the expression of lysosomal-associated membrane protein 1 (LAMP1), P450c17$\alpha$ (microsomal), and StAR (mitochondrial), all of which were found in their predicted subcellular fractions (Figure 5.1A). As expected, SF-1 is primarily localized in the nuclear envelope fraction with slight expression in the nucleoplasmic fraction. Confocal analysis confirmed that ASAH1 and SAP-D are expressed in the nuclei of H295R cells (Figure 5.1B).

### *5.3.2. $Bt_2$cAMP acutely stimulates ASAH1 activity*

Because cortisol biosynthesis is primarily regulated by the ACTH/cAMP pathway and ACTH signaling induces Cer turnover in adrenocortical cells (75), I next investigated the effect of ACTH/cAMP signaling on ceramidase activity. H295R wild type (WT) cells were incubated with 4 $\mu$M NBD-12-Cer for 4 h and then treated with 0.4 mM $Bt_2$cAMP. Whole lysates were purified for *in situ* ASAH1 activity assays. As shown in Figure 5.2A, 30 min of $Bt_2$cAMP stimulation increased ceramidase activity by 6-fold. In order to determine the role of ASAH1 in $Bt_2$cAMP-stimulated ceramidase activity, $ASAH1^{KD}$ cells described in Chapter Four (Section 4.2) were used. Tet-treated (96 h) $ASAH1^{KD}$ cells were incubated with 4 $\mu$M NBD-12-Cer for 4 h and then treated with 0.4 mM $Bt_2$cAMP. As shown in Figure 5.2A, no increase in ceramidase activity was observed in response to $Bt_2$cAMP in $ASAH1^{KD}$ cells. In agreement with this finding, mass spectrometric analysis of nuclear Cer, SPH, and S1P concentrations in WT and $ASAH1^{KD}$ cells revealed that ASAH1 suppression resulted in an overall reduction in nuclear concentrations of all three sphingolipid species; the most significant decrease being in SPH levels which were reduced by 85% in $ASAH1^{KD}$ cells (Figure 5.2B). Furthermore, $Bt_2$cAMP induced SM and Cer turnover as well as SPH and S1P accumulation in WT

cells whereas no significant change in the nuclear amounts of these sphingolipid species was observed in ASAH1$^{KD}$ cells (Figure 5.2B).

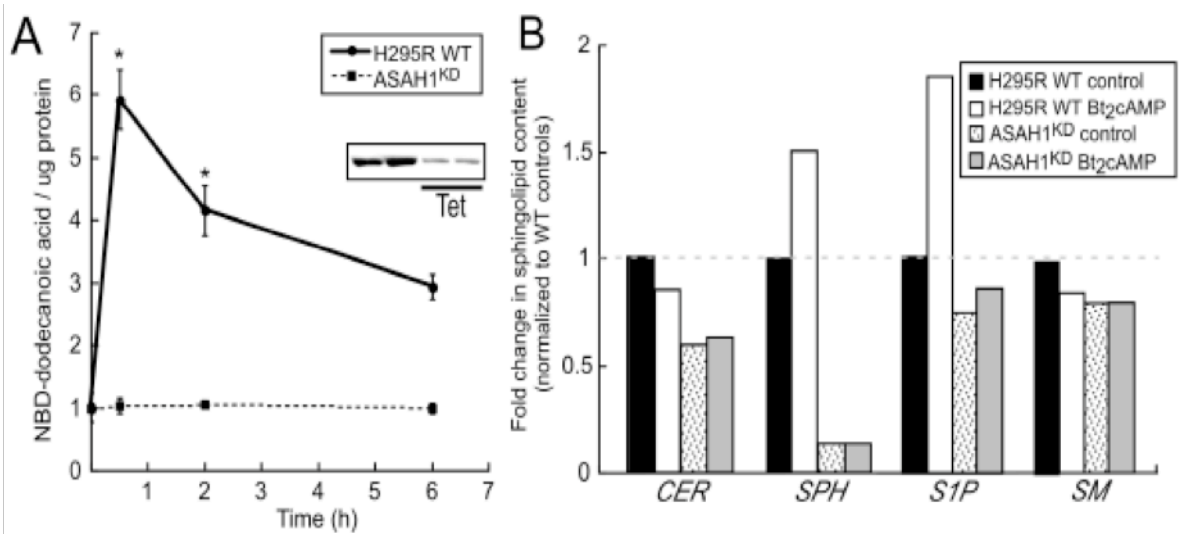



**Figure. 5.2.** *$Bt_2$cAMP increases nuclear ASAH1 activity*. **A**. H295R wild type (WT) and ASAH1$^{KD}$ (pre-treated for 96 h with 5 μg/mL tetracycline-tet) cells were treated for 30 min, 2, or 6 h with 0.4 mM $Bt_2$cAMP and nuclear extracts were isolated as described in Section 5.2. H295R WT or ASAH1$^{KD}$ nuclear extracts (50 μg) were incubated with 2 μl of 1 mM NBD-12-Cer in acetate buffer (pH 4.5) at 37°C for 2 h. Reactions were stopped and lipids were extracted and spotted on TLC plates. Plates were developed and visualized by fluorescent scanning. NBD-dodecanoic acid formation was quantified and normalized to the protein content of each sample. Data graphed represents mean ± SEM of 3 separate experiments, each performed in duplicate. *Inset:* Representative western blot of controls and tet-treated H295R$^{KD}$ cells demonstrating decreased ASAH1 protein levels. *Statistically different from untreated control group, $p < 0.05$. **B**. H295R WT or ASAH1$^{KD}$ (pre-treated for 96 h with 5 μg/mL tet) cells sub-cultured into 100-mm dishes and sphingosine (SPH), ceramide (CER), sphingomyelin (SM), and sphingosine-1-phosphate (S1P) concentrations in purified nuclei were quantified by LC-ESI-MS/MS using appropriate standards. Data graphed represents fold change in sphingolipid content and are normalized to WT untreated controls.

### *5.3.3. ASAH1 overexpression attenuates SF-1-dependent CYP17A1 reporter gene activity*

Next, I determined the role of ASAH1 in modulating CYP17A1 gene expression in response to ACTH/cAMP signaling by carrying out reporter gene assays using an ASAH1 expression plasmid and a CYP17A1 reporter gene construct. As previously shown, SF-1 increased both basal and $Bt_2$cAMP-stimulated CYP17A1 reporter gene activity by 2.4- and 9.8-fold, respectively (Figure 5.3A). ASAH1 suppressed $Bt_2$cAMP-

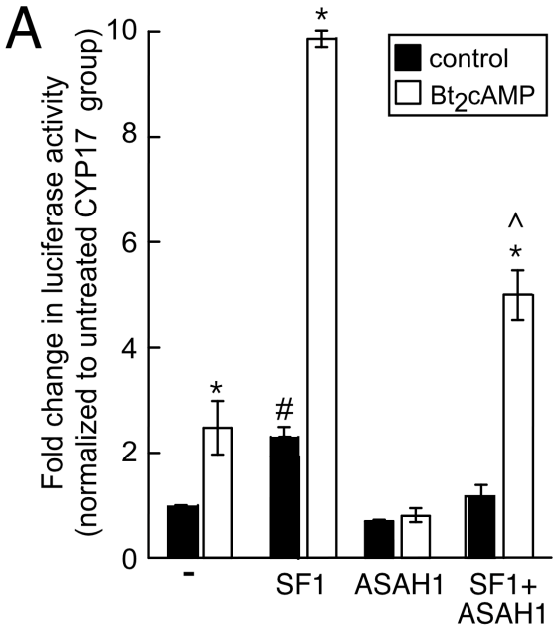
stimulated and SF-1-dependent CYP17A1 promoter activity in unstimulated and $Bt_2$cAMP-treated cells (Figure 5.3A).

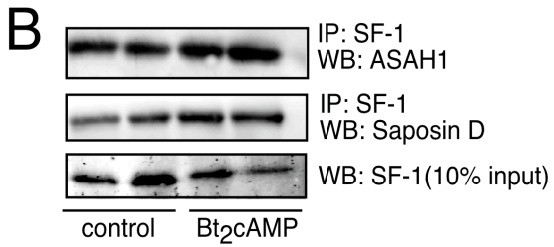
### *5.3.4. ASAH1 interacts directly with SF-1*

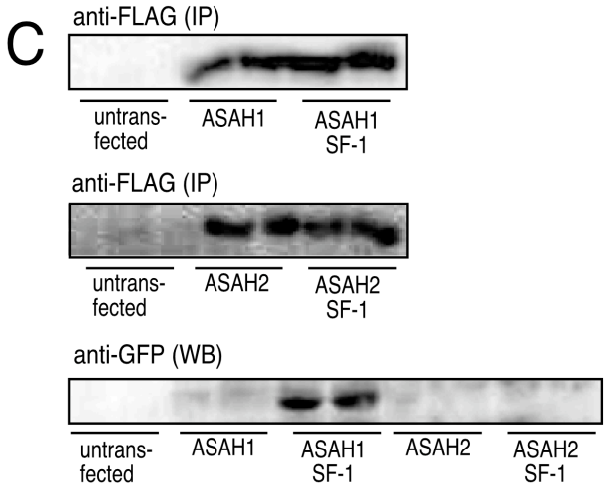
Based on the nuclear localization of ASAH1 (Figure 5.1), the effect of ASAH1 on SF-1-stimulated CYP17A1 reporter gene activity (Figure 5.3A), and the role of SPH as an antagonist for SF-1 (47), I hypothesized that ASAH1 modulates SF-1 activity by directly binding to the receptor. To test this hypothesis, coimmunoprecipitation assays were carried out, which revealed that endogenous ASAH1 and SAP-D co-immunoprecipitate with SF-1 (Figure 5.3B). Additionally, these proteins also interacted *in vitro* using GFP- and FLAG-tagged SF-1 and ASAH1 plasmids, respectively (Figure 5.3C). Importantly, ASAH2, which is also localized in the nuclei of H295R cells (Figure 5.1A), failed to interact with SF-1 (Figure 5.3C). To further investigate the interaction between SF-1 and ASAH1, *in vitro* pull-down assays using bacterially expressed His-tagged SF-1 and *in vitro* translated $^{35}$S-ASAH1 were carried out. As shown in Figure 5.3D (lower panel), SF-1 copurifies with radiolabelled ASAH1 (lane 3), confirming a direct interaction between the two proteins.

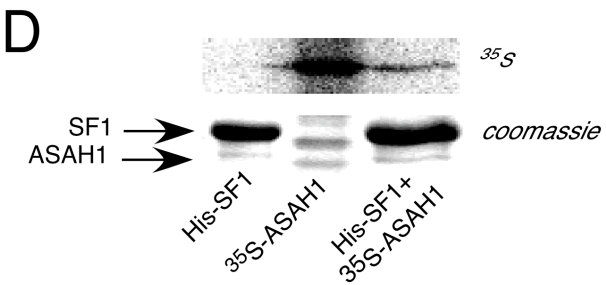
### *5.3.5. Mapping the ASAH1 regions that interacts with SF-1*

*In silico* analysis of ASAH1 revealed 7 putative LXXLL-related motifs (Figure 5.3E). To test whether ASAH1 binds to SF-1 through one of these motifs, site-directed mutagenesis was carried out to mutate these regions as described in Section 5.2.9. As shown in Figure 5.3F, ASAH1 harboring a mutation of LXXLL1 (NR1) (located in the $\alpha$ subunit of ASAH1) was unable to interact with the receptor, indicating that this region is required for mediating the binding of ASAH1 to SF-1. Mutation of LXXLL5 (located in the $\beta$ subunit of ASAH1) modestly reduced the interaction between ASAH1 and SF-1 but to




E

NR1
MNCCIGLGEKARGSHRASYPSLSALFTEASILGFGSFAVKAQWTEDCRKSTYPPSGPT
YRGAVPWYTINLDLPPYKRWHELMLDKAPVLKVIVNSLKNMINTFVPSGKIMQVVDEK
NR2 NR2x NR3
LPGLLGNFPGPFEEEMKGIAAVTDIPLGEIISFNIFYELFTICTSIVAEDKKGHLIHGRNMD
NR4 NR5
FGVFLGWNINNDTWVITEQLKPLTVNLDFQRNNKTVFKASSFAGYVGMLTGFKPGLFSL
NR6
TLNERFSINGGYLGILEWILGKKDVMWIGFLTRTVLENSTSYEEAKNLLTKTKILAPAYFIL
GGNQSGEGCVITRDRKESLDVYELDAKQGRWYVVQTNYDRWKHPFFLDDRRTPAKM
CLNRTSQENISFETMYDVLSTKPVLNKLTVYTTLIDVTKGQFETYLRDCPDPCIGW

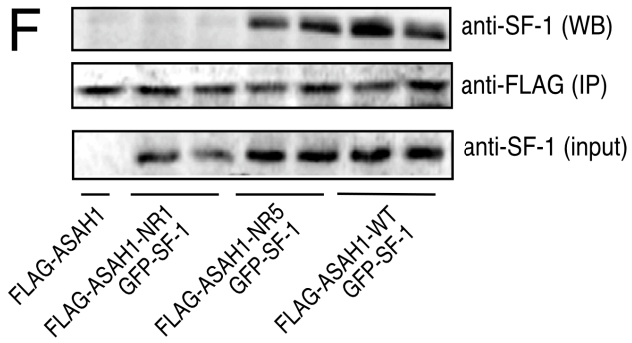


**Figure. 5.3.** *ASAH1 binds to SF-1*. **A.** H295R cells were transfected with pGL3-CYP17A1-2x57 and expression plasmids for ASAH1 and SF-1. Cells were then treated with 0.4 mM $Bt_2$cAMP for 16 h and harvested for dual luciferase assays. Data are expressed as fold increase in pGL3-CYP17A1 reporter gene activity and represents the mean ± SEM of 3 separate experiments, each performed in triplicate. Asterisks (*) and carats (^) denote statistically significant differences ($p < 0.05$) from untreated control group or treated SF-1-transfected group, respectively. **B.** H295R cells were treated with 0.4 mM $Bt_2$cAMP for 30 min and lysates were subjected to immunoprecipitation using an anti-SF1 antibody and protein A/G agarose. Immobilized proteins were washed, separated by SDS-PAGE, and analyzed by western blotting. Blots were hybridized to anti-ASAH1 or anti-saposin D antibodies (top two panels). Ten % inputs were subjected to SDS-PAGE and western blotting using an antibody against SF-1 (lower panel). **C.** H295R cells were transfected with 5 μg pCMV6-GFP-SF-1 and pCMV6myc/FLAG-ASAH1 or pCMV6myc/FLAG-ASAH2 expression plasmids and lysates subjected to IP with an anti-FLAG antibody and protein A/G agarose. Immobilized proteins were washed and separated by SDS-PAGE followed by western blotting analysis using an anti-GFP antibody (bottom panel). Ten % inputs were subjected to SDS-PAGE and western blotting using an antibody against FLAG (top and middle panels) **D**. His-tagged SF-1 was bacterially expressed from E. coli and ASAH1 was *in vitro* transcribed/translated from the pCMV6-XL5-ASAH1 vector. Twenty μg of *in vitro* translated $^{35}$S-ASAH1 was incubated with 50 μL His-tagged SF-1 in a tube rotator for 24 h at 4°C. SF-1/ASAH1 complexes were then subjected to SDS-PAGE and coomassie staining. Gel was dried, exposed to a radioactive-imager screen, and visualized by radioactive-imager scanning. **E.** Schematic representation of the putative LXXLL (NR)-related motifs (underlined/bold) in ASAH1. The β subunit sequence is underlined and putative NR motifs are highlighted. NR motifs in red were mutated using site-directed mutagenesis as described in Section 5.2. **F.** H295R cells were transfected with 10 μg pCMV6-GFP-SF-1 and pCMV6myc/FLAG-ASAH1 wild type (WT) or –LXXLL1 (NR1) or -LXXLL5 (NR5) mutant expression plasmids and lysates subjected to IP with an anti-FLAG antibody and protein A/G agarose. Immobilized proteins were washed and separated by SDS-PAGE followed by western blotting analysis using an anti-SF-1 antibody (top panel). Ten % inputs were subjected to SDS-PAGE and western blotting using antibodies against FLAG and SF-1 (lower panel).

a much lesser extent than LXXLL1 (Figure 5.3F). Consistent with this finding, reporter gene assays revealed that mutation of LXXLL1 rendered ASAH1 incapable of repressing SF-1/$Bt_2$cAMP-dependent CYP17A1 promoter activity (Figure 5.4A) whereas mutation of LXXLL5 in ASAH1 had no effect on the ability of the ceramidase to suppress SF-1 activity (Figure 5.4A).

In order to map additional interacting regions on ASAH1 that are involved in binding to SF-1, truncation mutants of ASAH1 were generated by cloning the primary genomic sequence of the $\alpha$ and $\beta$ subunits of ASAH1 into the pACT vector. Mammalian two-hybrid assays in H295R cells were then carried out to test the interaction between full-length, $\Delta\alpha$, or $\Delta\beta$ ASAH1 with SF-1. Consistent with coIP studies (Figure 5.3F), ASAH1$\Delta\alpha$ interacts with the receptor, but, surprisingly, ASAH1$\Delta\beta$ had a 2-fold stronger interaction for the receptor than ASAH1$\Delta\alpha$ (Figure 5.4B). This suggests that additional LXXLL motifs (or other regions) within the $\beta$ subunit of ASAH1 are also involved in mediating the binding of ASAH1 to SF-1.

Because the transcriptional potential of SF-1 is potently influenced by its interaction with various coregulatory proteins (158), reporter gene assays were carried out to determine the effect of ASAH1 expression on the ability of coregulators to modulate SF-1-dependent CYP17A1 promoter activity. As shown in Figure 5.4C, ASAH1 not only inhibited the ability of SRC-1 to potentiate SF-1-dependent promoter activity but also potentiated the repressory activity of SMRT. The ability of these coregulators to alter the interaction between ASAH1 and SF-1 was tested using mammalian 2-hybrid assays. Unexpectedly, SMRT significantly abrogated the interaction between full-length ASAH1 and SF-1 and completely prevented the interaction between ASAH1$\Delta\alpha$ and the receptor (Figure 5.4B). While SRC-1 strengthened the interaction between full-length

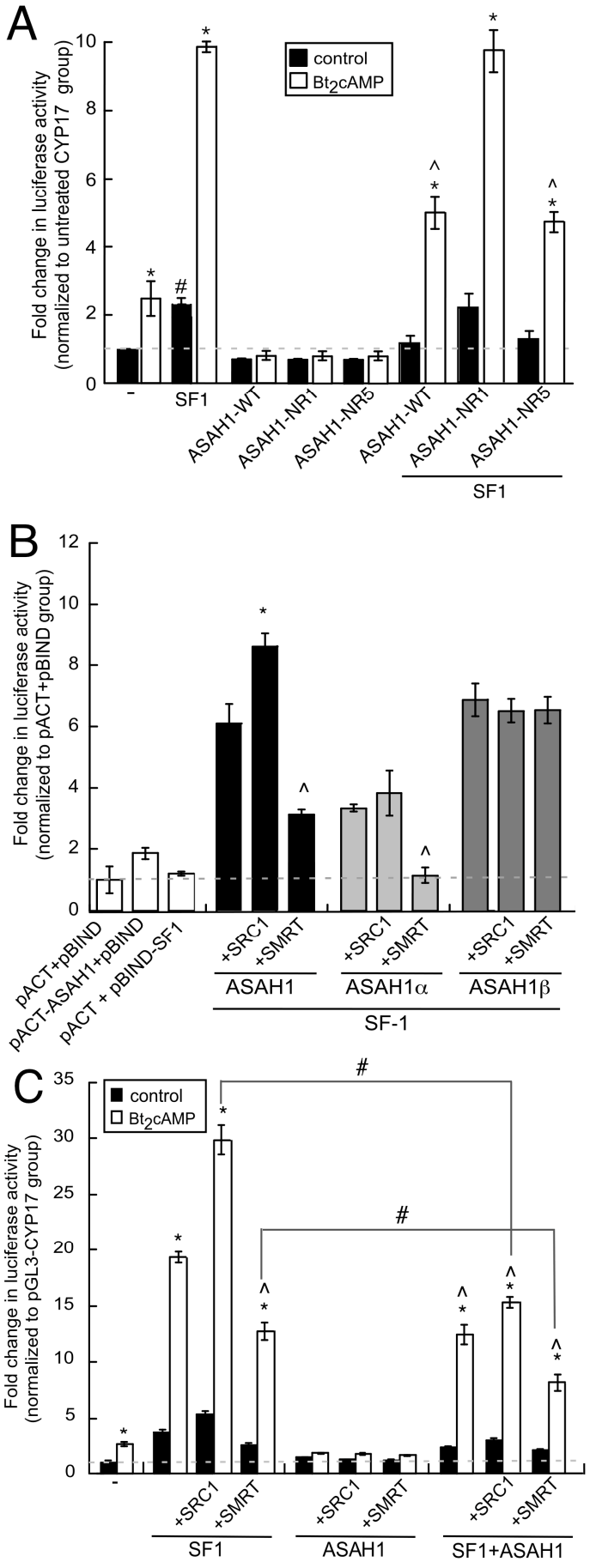



**Figure. 5.4.** *LXXLL-mediated ASAH1 binding to SF-1 affects coregulator activity.* **A.** H295R cells were transfected with pGL3-CYP17A1 and expression plasmids for SF-1, ASAH1-WT (wild type), ASAH1-NR1 (LXXLL1 mutant), or ASAH1-NR5 (LXXLL5 mutant). Cells were treated with 0.4 mM $Bt_2cAMP$ for 16 h and harvested for dual-luciferase assays. Data are expressed as fold increase in pGL3-CYP17A1 reporter gene activity and represent the mean ± SEM of 3 separate experiments, each performed in triplicate. (*), (^), and (#) denote statistically significant differences from untreated controls within each group, treated SF-1-transfected group, or untreated controls (-), respectively. **B.** H295R cells were transfected with pGL5luc, pACT, and pBIND (empty vectors) or pGL5luc, pBIND-SF-1 and pACT-ASAH1, pACT-ASAH1Δα, or pACT-ASAH1Δβ plasmids in the presence or absence of expression plasmids for SRC-1 or SMRT. Transfected cells were harvested after 48 h and luciferase activity quantified by luminometry. Data graphed represents the mean ± SEM of at least 3 separate experiments, each performed in triplicate. **C.** H295R cells were transfected with pGL3-CYP17A1 and expression plasmids for SF-1, ASAH1, SRC-1, and SMRT. Cells were treated with 0.4 mM $Bt_2cAMP$ for 16 h and harvested for dual-luciferase assays. Data are expressed as fold increase in pGL3-CYP17A1 reporter gene activity and represent the mean ± SEM of 3 separate experiments, each performed in triplicate. (*), (^), and (#) denote statistically significant differences from untreated controls within each group, treated SF-1-transfected group, or as shown in figure, respectively.

ASAH1 and SF-1 by 1.4-fold, neither coregulator affected the interaction between ASAH1Δβ and SF-1 (Figure 5.4B).

### *5.3.6. The catalytic activity of ASAH1 is required for its repressory function*

To determine whether ceramidase activity (SPH production) is required for the repressive effect of ASAH1, H295R cells were pre-treated with the ceramidase inhibitor D-NMAPPD (485) and the ability of ASAH1 to inhibit SF-1 activity was assessed in reporter gene assays. As shown in Figure 5.5, inhibiting the catalytic activity of ASAH1 rendered the ceramidase unable to suppress SF-1/$Bt_2$cAMP-induced CYP17A1 reporter gene activity in a dose-dependent manner.

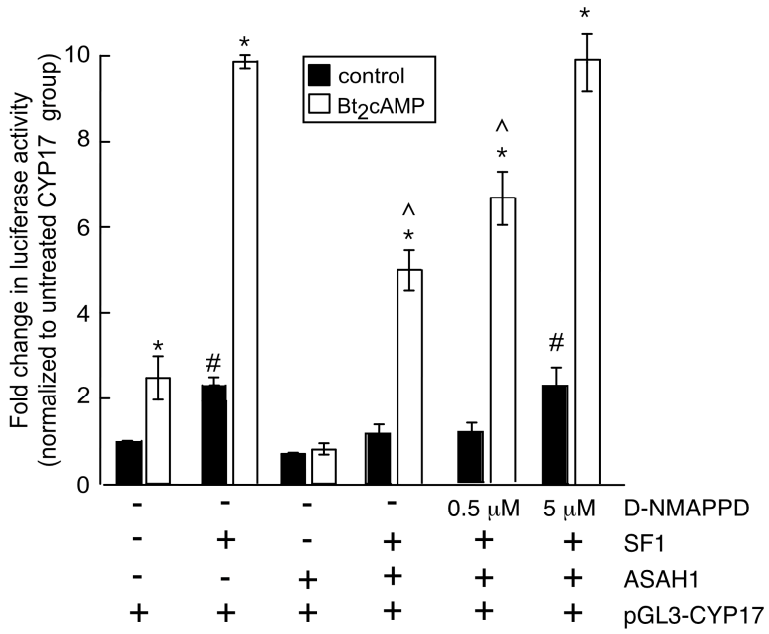



**Figure 5.5.** *A catalytically active ASAH1 is required for suppression of SF-1 activity.* H295R cells were transfected with pGL3-CYP17A1 and expression plasmids for SF-1 and ASAH1. Cells were pre-treated for 1 h with 0.5 μM or 5 μM D-NMAPPD followed by treatment with 0.4 mM $Bt_2$cAMP for 16 h. Cells were then harvested for dual-luciferase assays. Data are expressed as fold increase in pGL3-CYP17A1 reporter gene activity and represent the mean ± SEM of 3 separate experiments, each performed in triplicate. (*), (^), and (#) denote statistically significant differences ($p < 0.05$) from untreated controls within each group, treated SF-1-transfected group, or untreated controls (-), respectively.

### *5.3.7. $Bt_2$cAMP promotes the recruitment of ASAH1 to multiple steroidogenic gene promoters*

To examine the recruitment of ASAH1 to the endogenous CYP17A1 promoter, ChIP assays were carried out using chromatin isolated from H295R cells treated with 0.4 mM $Bt_2$cAMP for 30 min. The primer set used amplifies the promoter region -104/+43, which contains a key ACTH/cAMP-responsive SF-1 binding site at -57/-37 (158). Elevated ASAH1 occupancy on the promoter was observed after $Bt_2$cAMP stimulation, which correlated with the relative enrichment of SF-1-bound CYP17A1 promoters (Figure 5.6A). Consistent with transcriptional initiation, $Bt_2$cAMP promoted the recruitment of RNA Polymerase II and increased the levels of tri-methylated histone H4 at lysine 20 (H4K20). Importantly, neither ASAH2 nor ACER3 were detected in this region of the CYP17A1 promoter under these conditions (data not shown). To investigate whether ASAH1 and SF-1 occupy the same region of the promoter, sequential immunoprecipitation (reChIP) assays were carried out. As shown in Figure 5.6B, these two proteins exist as a complex on the CYP17A1 and StAR promoters. Interestingly, the pattern of complex formation appears to be promoter-specific. $Bt_2$cAMP stimulation induced ASAH1/SF-1 complex formation at the CYP17A1 promoter (Figure 5.6B, left panel) while it reduced protein-protein interaction at the StAR promoter (Figure 5.6B, right panel). Finally, to examine the recruitment of ASAH1 to additional SF-1-regulated promoters, DAX-1 and Mc2R, ChIP assays were carried out. The primers used were designed to amplify the proximal promoter region of each gene and encompass at least one SF-1 binding site. As shown in Figure 5.6C, $Bt_2$cAMP stimulation promoted the recruitment of ASAH1 to both promoters, but the fold change in ASAH1 recruitment, compared to SF-1 enrichment, was promoter-specific. At the Mc2R promoter, ASAH1 occupancy was 3.1-fold higher than SF-1 under basal conditions and $Bt_2$cAMP stimulation promoted a 12-fold increase in SF-1 enrichment compared to a 2.5-fold

increase in ASAH1 occupancy (Figure 5.6C, left panel). Conversely, both SF-1 and ASAH1 had equivalent levels of occupancy at the DAX-1 promoter in unstimulated cells, but the fold-increase in ASAH1 enrichment (56-fold) in response to $Bt_2$AMP was significantly higher than SF-1 recruitment (5-fold) (Figure 5.6C, right panel).

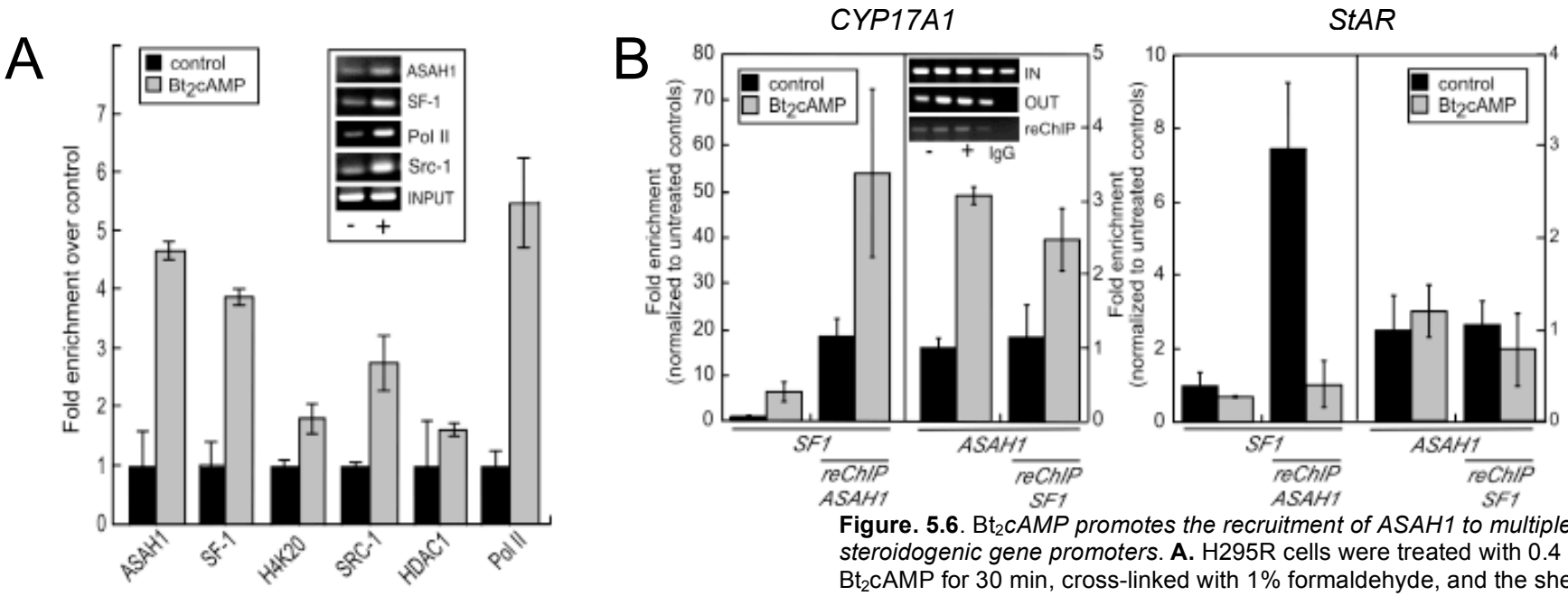



**Figure. 5.6**. $Bt_2$*cAMP promotes the recruitment of ASAH1 to multiple steroidogenic gene promoters*. **A.** H295R cells were treated with 0.4 mM $Bt_2$cAMP for 30 min, cross-linked with 1% formaldehyde, and the sheared chromatin immunoprecipitated (IP) using antibodies against SF-1, ASAH1, SRC-1, trimethyl-H4K20, RNA Pol II, and HDAC1. The relative amount of IP promoter fragments was normalized to their respective levels in the input DNA. *Inset:* representative agarose gels of the PCR products obtained for output and input DNA. **B.** H295R cells were treated with 0.4 mM $Bt_2$cAMP for 30 min, cross-linked with 1% formaldehyde, and reChIP assays were carried out as described in Section 5.2. The IP DNA fragments were analyzed by qPCR with primers spanning the proximal promoter regions (containing at least one SF-1 binding site) of CYP17A1 (left panel) and StAR (right panel) genes. The relative amount of IP promoter fragments was normalized to their respective levels in the input DNA. *Inset on left panel:* representative agarose gels of the PCR products obtained for input DNA (IN, top panel), ASAH1-bound (OUT, middle panel), and reChIP for SF1 (reChIP, bottom panel) chromatin. **C.** H295R cells were treated with 0.4 mM $Bt_2$cAMP for 30 min, cross-linked with 1% formaldehyde, and the sheared chromatin IP using antibodies against SF-1 or ASAH1. The IP DNA fragments were analyzed by qPCR with primers spanning the proximal promoter regions of DAX-1 and Mc2R genes. The relative amount of IP promoter fragments was normalized to their respective levels in the input DNA and is graphed as fold enrichment over untreated SF-1-immunoprecipitated DNA.

## 5.4. Discussion

A growing body of literature supports the role of sphingolipid metabolism in the regulation of steroid hormone production (440). Our group has previously identified the role of SPH as an antagonist for SF-1 (47). However, because the mechanisms that control ligand availability are unknown, I sought to determine the role of ASAH1, a SPH-generating enzyme, in regulating SF-1 function. The results presented in this Chapter demonstrate that ASAH1 is expressed in the nucleus of H295R cells and its activity is acutely regulated by ACTH/cAMP signaling. ASAH1 represses the transcriptional potential of SF-1 by directly binding to the receptor on the promoter of multiple steroidogenic genes. Significantly, the interaction between ASAH1 and SF-1 occurs through a functional LXXLL-related motif, which is essential for conferring ASAH1-dependent repression of SF-1.

Mounting evidence has linked multiple sphingolipid species to various nuclear processes (reviewed in (444)). Due to the hydrophobic nature of most sphingolipids, the nuclear expression of sphingolipid enzymes suggest that there is a dynamic and localized production of bioactive lipids, which may have unique roles in nuclear processes that are independent from their cytoplasmic functions. The present study revealed that ASAH1 and its coactivator protein SAP-D are expressed in the nucleus of H295R cells (Figure 5.1), which expands the likelihood of local sphingolipid metabolism in these cells. In fact, mass spectrometric analysis revealed that ACTH/cAMP signaling promotes rapid nuclear SM and Cer turnover with a concomitant accumulation of SPH and S1P in an ASAH1-dependent manner (Figure 5.2).

Because ASAH1 was characterized as a lysosomal protein, the nuclear localization of ASAH1 represents a novel concept. Nuclear import of proteins is usually based on the presence of a basic amino acid sequence designated as the nuclear localization signal (NLS), which mediates binding to importins and translocation through

the nuclear pore complex (NPC) (486). In addition to this 'classical' mechanism of protein nuclear import, other pathways for the transport of non-NLS-containing proteins into the nucleus have been described. One such mechanism is the nuclear import of glycoproteins via a mechanism that utilizes sugar-binding carrier proteins (e.g. lectins) (487). Given that ASAH1 contains no NLS sequences but is glycosylated at multiple residues during processing (99), it is possible that sugar-binding proteins mediate its nuclear import in H295R cells. Alternatively, it was recently shown that nuclear import of ERK, which does not contain a canonical NLS, is mediated by casein kinase II-dependent phosphorylation of three residues (488), which promote ERK binding to importin 7 and translocation through NPCs (489). Notably, probing the subcellular localization of ASAH1 in other cell lines revealed that this ceramidase is nuclear in murine Y-1 adrenocortical cells whereas it is cytoplasmic in MCF-7 breast cancer cells (Lucki and Sewer, unpublished observations), suggesting that the nuclear functions of ASAH1 are cell-type specific.

The transcriptional activity of nuclear receptors is influenced by their ability to interact with numerous coregulator proteins. Conserved sequences containing a nuclear receptor (NR) motif of LXXLL (where X is any amino acid and L is a leucine that can be replaced by an isoleucine, methionine, or phenylalanine) (168-170,174), LXX-(I/H)-IXXX-(I/L) helix (490), or (L/I)-XX-(I/V)-I (CoRNR) box (491) have been implicated in receptor-corepressor interaction. Studies described in this Chapter demonstrate that ASAH1 is a novel coregulator of SF-1 that represses receptor activity by a direct protein-protein interaction involving an LXXLL-related motif in the $\alpha$ subunit of ASAH1. Site directed mutagenesis revealed that this motif is required not only for interaction with the receptor (Figure 5.3F) but also for the enzyme to repress SF-1 activity (Figure 5.4A). The fact that ASAH1 co-localizes with SF-1 on the promoter of the CYP17A1 and StAR genes (Figure 5.6B) is consistent with a coregulator function. Coregulators are recruited to DNA by

nuclear receptors and either have intrinsic catalytic properties or recruit enzymatic protein complexes (e.g. HDAC, histone methyltransferases or acetyltransferases) to mediate chromatin and/or nuclear receptor modifications that influence gene transcription. Coactivators enhance nuclear receptor-mediated transcription while corepressors repress gene expression, primarily through their interaction with unliganded nuclear receptor (492).

The molecular determinants of SF-1 interaction with various coregulators, such as Dax-1 (122,493-494), SRC-1 (119), GRIP1 (149), peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$ coactivator 1$\alpha$ (PGC-1$\alpha$) (495), and DGK$\theta$ (164), have been described. Suzuki *et al.* (493) and Zhang *et al.* (170) proposed a model for Dax-1-dependent SF-1 or ER$\alpha$ transcriptional repression, respectively, where Dax-1 binding to nuclear receptor competes with coactivators (e.g. SRC-1) as well as recruits corepressors (e.g. NCoR). Because my data show that ASAH1 expression prevented SRC-1 from upregulating SF-1-dependent CYP17A1 promoter activity while potentiating SMRT repressory activity, it is possible that the underlying mechanism by which ASAH1 suppresses SF-1 function is similar to Dax-1.

Significantly, pharmacological inhibition of ASAH1 rendered the enzyme incapable of repressing SF-1. Collectively, these findings suggest that the co-repressive function of ASAH1 requires not only an interaction with SF-1 but also a catalytically-competent enzyme. This premise is consistent with my hypothesis that ASAH1 controls SF-1 function by regulating ligand (i.e. SPH) availability, although *in vivo* and *in vitro* biophysical studies of the ligand-binding event are necessary to fully prove this theory. Notably, our group has previously reported an LXXLL-dependent interaction between SF-1 and DGK$\theta$ (164), the enzyme that produces the SF-1 agonist PA. DGK$\theta$ activity and binding to SF-1 are required events for $Bt_2$cAMP-dependent CYP17A1 mRNA expression (164). My current findings lend support to the reciprocal regulation of SF-1 by

ASAH1 is response to ACTH/cAMP signaling where ASAH1 binding to SF-1 represses receptor activity possibly by facilitating ligand exchange (i.e. the exchange of PA for SPH) and/or promoting corepressor recruitment (e.g. SMRT) (Figure 5.7). It is counter-intuitive to think that activation of the ACTH/cAMP signaling cascade, which is the main activator of steroidogenic gene transcription, would stimulate ASAH1 DNA recruitment and SF-1 binding. However, because SF-1-containing transcriptional complex assembly at target promoters is dynamic and cyclical, as demonstrated by Winnay *et al.* (161) and our group (158), it is probable that ACTH evokes a spatio-temporal transcriptional regulatory mechanism where a cyclic, reciprocal exchange of coactivators and corepressors ensures optimal target gene transcription. In addition, because SPH is an activator of DGK (496-497), it is also possible that ACTH/cAMP-dependent nuclear ASAH1 acitivity plays a role in controlling DGKθ activity. Temporal ChIP experiments will be imperative to dissect the temporal order of DGKθ and ASAH1 DNA recruitment during SF-1-mediated transcription. In addition, determining whether ASAH1 promoter recruitment is dependent on SF-1 expression or vice-versa is central to fully understand the inter-regulation between these two proteins. These studies are currently being undertaken.

Notably, the ability of various nuclear receptors, including estrogen receptor (ER) β (498), PPARγ (499), and SF-1 (121), to interact with coregulatory proteins is regulated by phosphorylation. Therefore, it is possible that post-translational modifications of SF-1 may be involved in temporally regulating the receptor's affinity for ASAH1 in response to ACTH stimulation. Phosphorylation of SF-1 at $Ser^{203}$ enhances the receptor's ability to interact with GRIP1 and SMRT (121). Because these coregulators play opposite roles in gene transcription, additional layers of regulation as well as the recruitment of other coregulators (e.g. histone methyltransferases, acetyltransferases), possibly ASAH1, dictates the nature of the complex formed (e.g. coactivator or corepressor) and

transcriptional outcome (e.g. activation or repression). In summary, experiments in this Chapter defined the role of ASAH1 as a corepressor of SF-1 in H295R adrenocortical cells.

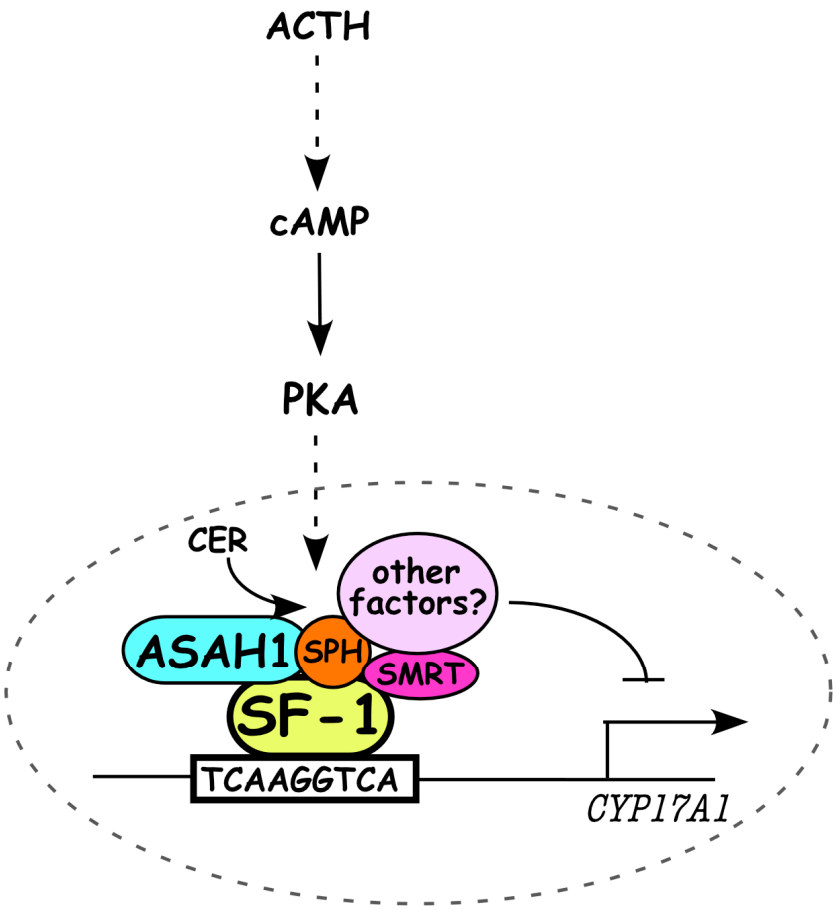



**Figure 5.7.** *Schematic model of the mechanism of ASAH1 repression of SF-1.* ACTH/cAMP signaling promotes ASAH1 recruitment to SF-1-target promoter, where it interacts with SF-1 through a functional LXXLL motif. SF-1 binds to its cognate response element on DNA upon ACTH stimulation. ASAH1 binding to the receptor suppresses its transcriptional function on the CYP17A1 promoter by producing the antagonist SF-1 ligand sphingosine (SPH). ASAH1 may also mediate the recruitment and/or potentiate the activity of the corepressor SMRT (silencing mediator for retinoid or thyroid-hormone receptors). Other factors (e.g. HDACs, histone methyltransferases) may stabilize or modify the interaction among these proteins, depending on the promoter context.

# CHAPTER 6:
*Conclusions*

## 6.1. ASAH1 as a key regulatory factor in adrenocortical steroidogenesis

Sphingolipids have a multitude of biological functions. The amounts of different sphingolipid species within a cell are controlled by a series of enzymes that are regulated by multiple factors. Therefore, the sphingolipid metabolic profile in a cell at any given point in time is comprised of a unique set of bioactive sphingolipids that are dynamically changing in response to changes in the intracellular and extracellular environments. Cer, SPH, and S1P regulate gonadal and adrenal steroidogenesis by participating in signaling cascades to control steroidogenic gene expression and steroid hormone output (51,75,188,238,482). The intracellular concentrations of these metabolites are regulated by the actions of multiple enzymes, including ceramidases. These enzymes play an integral role in maintaining proper cellular function not only by controlling SPH and S1P levels but also limiting the signaling properties of Cer. Our laboratory has previously demonstrated that SPH antagonizes the transactivation potential of SF-1 and shown that suppression of ASAH1 expression mimics ACTH/cAMP-dependent CYP17A1 transcription in H295R cells, thus suggesting a role for ASAH1 in the regulation of adrenocortical steroidogenesis. Furthermore, we have found that ACTH rapidly changes the sphingolipid profile of adrenocortical cells, whereby complex sphingolipids (i.e. SM and Cer) are catabolized and S1P is produced.

Although the precise mechanism by which ACTH regulates sphingolipid metabolism is unclear, our previous findings suggest that the dynamic changes in bioactive sphingolipids elicited by ACTH are important in the regulation of steroid hormone biosynthesis. Therefore, I hypothesized that ASAH1 is an effector molecule in the ACTH/cAMP signaling pathway. Furthermore, because ASAH1 controls the intracellular concentrations of SPH, a ligand for SF-1 (115), I postulated that ASAH1

modulates steroidogenic gene transcription by controlling the transcriptional activity of SF-1. Defining the molecular mechanisms underlying ASAH1 function in ACTH-dependent adrenocortical steroidogenesis was the central aim of this dissertation.

In the studies detailed in Chapters Two, Three, Four, and Five the following questions were addressed: 1) What is the role of S1P in the regulation of acute cortisol biosynthesis? 2) Does ACTH/cAMP signaling regulate ASAH1 transcription? If so, what transcriptional factors control promoter activity? 3) What is the effect of ASAH1 suppression on global gene expression, sphingolipid metabolism, and steroidogenic capacity of adrenocortical cells? 4) How does ASAH1 modulate the transactivation potential of SF-1?

In Chapter Two, I characterized the molecular mechanism by which S1P, the bi-product of ASAH1 activity, activates HSL and induces StAR expression, which culminates in the upregulation of cortisol synthesis. Chapter Three focused on the functional characterization of the ASAH1 promoter and established CREB as an essential transcriptional regulator of the ASAH1 gene. Moreover, I found that the ACTH/cAMP pathway chronically induces ASAH1 protein expression and enzymatic activity. In Chapter Four, a novel function of ASAH1 as a global regulator of adrenocortical gene expression was uncovered. With the use of an ASAH1 knockdown adrenocortical cell line, I demonstrated that ASAH1 expression is essential for optimal transcription of multiple steroidogenic and nuclear receptor genes, all of which are important for cholesterol transport and/or steroid hormone metabolism. Accordingly, suppressing ASAH1 altered the amount of cortisol and DHEA produced by these cells. I further showed that ASAH1 silencing altered the cellular amounts of acetylated histone H3, which suggests that ASAH1 plays a role in the epigenetic regulation of gene expression. Consistent with these findings, experiments in Chapter Five identified

ASAH1 as a nuclear protein in H295R cells and characterized its role as a corepressor of SF-1.

The overall conclusions of the studies presented in this dissertation are: (1) S1P is a pleiotropic regulatory factor of adrenocortical function that mediates cholesterol transport, and cortisol production, by promoting HSL activation and inducing StAR expression. (2) ACTH/cAMP signaling promotes CREB-dependent transcription of the ASAH1 gene. (3) ASAH1 is a negative regulator of steroidogenic gene expression and adrenocortical steroidogenesis. (4) ASAH1 is a novel SF-1 corepressor in adrenocortical cells.

### 6.2. The role of S1P as a paracrine factor in adrenocortical cells

The versatility of S1P-mediated cellular responses arrises from the fact that this metabolite not only acts as an intracellular second messenger but also is secreted from cells and activates signaling through multiple cell-surface S1PRs (Figure 1.8). S1P is an intermediate linking various extracellular factors and their respective biological functions. For example, S1P mediates various TNF-$\alpha$-dependent pro-inflammatory effects, including priming of neutrophils (500) and NF-$\kappa$B activation (501). Similarly, prolactin (502) and 17$\beta$-estradiol ($E_2$) (192) promote breast cancer cell growth through the upregulation of SPHK activity and S1P production. Furthermore, it was recently shown that the adipocyte-derived secretory factor adiponectin promotes cell survival by triggering S1P production (503). Given the pleiotropic functions of S1P and the array of factors that modulate its production, experiments described in Chapter Two were focused on defining the role of S1P during the acute phase (Section 1.1) of ACTH-dependent steroidogenesis.

Previous work form our laboratory has established S1P as a secondary factor to ACTH in the upregulation of CYP17A1 gene transcription (115). The intricate molecular

details of this regulation include rapid ACTH-stimulated S1P production and secretion, and S1PR-dependent cleavage and nuclear translocation of the transcription factor SREBP1. Once in the nucleus, SREBP1 binds to the CYP17A1 promoter and drives gene transcription (115). ACTH/cAMP signaling activated SPHK after 15 min and increased extracellular S1P was seen 30 min after stimulation. The effect of S1P on SREBP1 cleavage and CYP17A1 transcription were both time-dependent: maximal SREBP1 maturation (i.e. cleavege) was observed 4 h after S1P stimulation while increased CYP17A1 mRNA expression was seen after 6 h.

The findings of the present work expand the role of S1P as a regulator of adrenocortical cell function by characterizing the mechanism by which S1P acutely increases cortisol production. S1P acutely induces the transcription of multiple cholesterol transport-related genes, including StAR, TSPO, LDLR, and SR-BI (Figure 2.2), and rapidly stimulates the phosphorylation (i.e. activation) of HSL (Figure 2.6). Induction of LDLR and SR-BI expression increase the import of cholesteryl esters. HSL catalyzes the obligatory cleavage of imported cholesteryl esters, and StAR and TSPO facilitate movement of free cholesterol into the inner mitochondrial membrane, where its metabolism into steroid hormone begins (Section 1.1). Significantly, the current work identified S1P as a novel regulator of StAR gene expression via the activation of the MAPK/ERK pathway. Because StAR transcription is regulated by the bZIP family of transcription factors (e.g. CREB) (22,504) and S1P mediates CREB activitation (505), it is likely that CREB is recruited to the StAR promoter in response to S1P stimulation. Furthermore, given that sphingolipid-mediated SREBP1 cleavage (506) has been shown to mediate StAR transcription (507) and previous work from our group shows that S1P activates SREBP1 in H295R cells (115), it is plausable that SREBP1 is also recruited to the StAR promoter in response to S1P stimulation in H295R cells. Given the essential role of StAR in inner mitochondrial cholesterol import (the rate-limiting step in steroid

hormone production), my findings lend support to the integral role of S1P in maintaining optimal steroid hormone output during the acute phase of steroid hormone biosynthesis (Section 1.1). Collectively, we can envision S1P as a multi-functional stimulator of adrenal steroidogenesis: it indirectly modulates acute cholesterol transport into the inner mitochondrial membrane by regulating HSL activity and StAR expression (Chapter Two) while promoting chronic cholesterol metabolism by inducing CYP17A1 gene expression (115).

There are multiple potential implications for the actions of S1P in adrenocortical cells. Because S1P is secreted from some cell types, pathophysiological conditions that promote dysregulated S1P production/secretion often have a systemic effect and, therefore, may affect adrenal function. For example, sphingolipid metabolism in adipose tissue and plasma S1P levels are increased in genetically obese mice (508), suggesting that S1P-dependent cortisol production may be a catalyst for obesity. Similarly, S1P concentrations are also increased in a mouse model of diabetic nephropathy (509). Mast cells and platelets are known to upregulate the generation of S1P following immune stimulation (510-511). Hence, increased plasma concentrations of S1P may serve to damper or adjust the immune response by promoting the secretion of cortisol. Concurrently, persistent activation of the inflammatory response is associated with the etiology of cancer (reviewed in (512)) and many tumors are characterized by an overexpression of SPHK1 (513-518). As a result, S1P secreted from cancer cells due to hyperactive SPHK1 not only exerts tumorigenic effects (i.e. promotion of cell motility and growth, angiogenesis, etc.) but may also affect, in a systemic level, cortisol production. This is an intriguing premise because it suggests that S1P produced as a bi-product of inflammation not only acts to regulate the inflammatory response in immune cells, which is well established (519), but may also modulate inflammation by promoting cortisol biosynthesis. Consistent with this theory, studies have shown that glucocorticoids protect

cells from apoptosis through S1P-mediated mechanisms (520-523), suggesting a mutual relationship between S1P and glucocorticoids.

### 6.3. ACTH/cAMP-dependent transcriptional regulation of the ASAH1 gene

Most sphingolipid-related research has focused on the effects of these bioactive lipids on cellular processes, with transcriptional regulation of sphingolipid-metabolizing enzymes being less studied. However, based on a series of recent studies, it is becoming increasingly evident that regulation of sphingolipid gene expression is an integral mechanism by which various physiological factors control sphingolipid metabolism. In the present work, I show that ACTH/cAMP signaling chronically induces transcription of the ASAH1 gene (Figure 3.1), concomitant with an increase in protein expression (Figure 3.7). I also established CREB as an essential activator of this gene (Figure 3.4). To date, promoter analysis of some sphingolipid genes have been reported, including CERK (223), SPL (524), SGPP2 (222), SPTLC2 (419), ganglioside GM3 synthase (420), Cer glucosyltransferase (421), neutral SMase2 (525), and ASAH2 (422).

Park *et al.* (425) has previously defined the structural units of the murine ASAH1 gene and demonstrated that KLF6, Sp1, NF-κB, and AP2 can bind the promoter *in vitro*. However, prior to the present work, no functional characterization of this promoter has been reported. To this end, I demonstrate the binding of CREB, and the subsequent recruitment of the coactivator CBP/p300, to multiple regions of the human ASAH1 promoter (Figure 3.6). Importantly, the indispensable role of CREB in mediating ACTH/cAMP-induced ASAH1 mRNA expression was established by selectively suppressing CREB expression by RNAi (Figure 3.4) and by utilizing a dominant-negative expression plasmid (Figure 3.3). Of note, CREB has also been previously implicated in the regulation of the CERK gene in JEG-3 human placental choriocarcinoma cells (526). Although the physiological significance of CREB-dependent CERK regulation is

unknown, it suggests that ACTH/cAMP signaling regulates the transcription of multiple sphingolipid genes.

*In silico* analysis of the ASAH1 promoter revealed putative binding sites for additional transcription factors, such as ER and Sp1. In fact, I have shown that, in MCF-7 breast cancer cells, ERα and Sp1 bind to the ASAH1 promoter and induce gene transcription in response to stimulation with the phytoestrogen genistein (Figure 6.1). A detailed description of this regulation is presented in Appendix 1. Interestingly, the ASAH1 promoter also contains a putative binding site for the glucocorticoid receptor (GR) and treatment of adrenocortical cells with the synthetic glucocorticoid dexamethasone induces ASAH1 mRNA expression (Lucki *et al.,* upublished observation) (Figure 6.1). Dexamethasone-induced ASAH1 expression was previously reported in mice muscle tissue (527). Therefore, it is possible that ASAH1 is part of a local negative feedback loop that controls cortisol production. Of note, dexamethasone has also been shown to upregulate SPHK1 activity and ASAH2 expression in rat

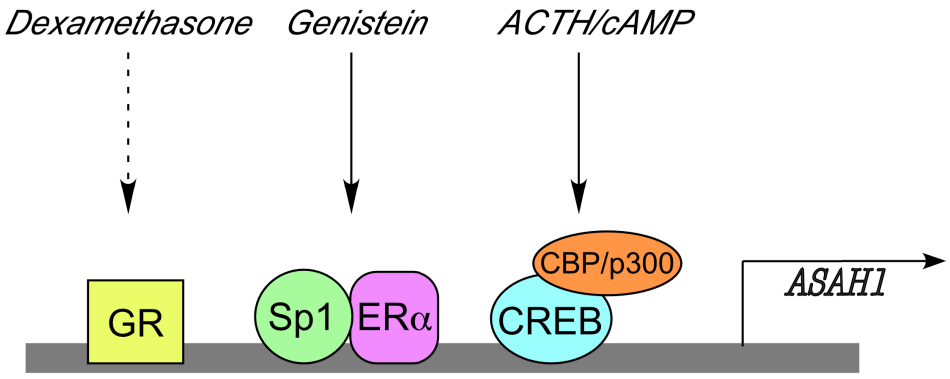



**Figure 6.1.** *ASAH1 promoter regulation by various transcription factors.* ACTH/cAMP, genistein, and dexamethasone induce ASAH1 mRNA expression. Arrows indicate the transcription factors that are recruited to the ASAH1 promoter in response to each stimulus. Solid arrows indicate that protein association with DNA has been reported whereas the dashed arrow indicates that transcription factor DNA recruitment is yet to be established. *Abbreviations:* glucocorticoid receptor (GR), specificity protein 1 (Sp1), estrogen receptor α (ERα), cAMP response element binding protein (CREB), CREB binding protein (CBP/p300).

mesangial cells (521), thus suggesting an intricate relationship between glucocorticoids and SPH/S1P levels.

Because CREB regulates the expression of numerous genes, it is possible that additional extracellular cues that trigger intracellular cAMP production may also influence ASAH1 gene expression. For example, PMA- (528) and insulin-like growth factor 1-stimulated (529) StAR gene transcription are dependent on the activation of CREB through the PKC pathway. Interestingly, as stated previously, S1P promotes CREB phosphorylation (i.e. activation) through the activation of the MAPK signaling cascade (505). Likewise, S1P inhibits cAMP production by activating signaling through $G\alpha_i$-coupled S1PRs (197-199). In theory, these pathways would reciprocally influence ASAH1 gene transcription. The possibility that S1P may play a role in ASAH1 transcription is relevant to the context of the present work because this would imply that S1P modulates cortisol biosynthesis not only by inducing StAR (Chapter Two) and CYP17A1 (115) transcription but also by regulating ASAH1 gene expression. Given that S1P is formed by the action of ASAH1, this suggests a potential feed-forward mechanism between S1P signaling and ASAH1 expression. The intricate details of this potential crosslink between S1P signaling and ASAH1 expression are yet to be investigated. Nonetheless, it is evident that the range of possible sphingolipid-mediated regulatory mechanisms of adrenocortical steroidogenesis is vast and a lot of interconnections are likely to exist.

### 6.4. ASAH1 as a global regulator of adrenocortical gene transcription

As discussed previously, nuclear sphingolipid metabolism is a fairly recent concept, but emerging research is uncovering many roles for sphingolipids in nuclear processes. However, the concept of sphingolipid-metabolizing enzymes serving as transcriptional regulators is largely unexplored. Arguably, the most significant

demonstration of sphingolipid-mediated transcriptional regulation was recently provided by Hait *et al.* (255). The authors revealed a novel role for nuclear SPHK2 as an inhibitor of HDAC1/2 and an epigenetic regulator of gene expression in MCF-7 breast cancer cells. SPHK2-generated S1P binds to HDAC1/2 and inhibits deacetylase activity, resulting in increased histone H3 acetylation and upregulation of $p21^{CIP1/WAF1}$ gene expression (255). Interestingly, in contrast to the effect of S1P, $C_{18}$-Cer was shown to activate HDAC (530-531). My data suggest that ASAH1 also modulates post-translational modification of histones because suppression of ASAH1 expression led to increased levels of acetylated histone H3 at the proximal promoter region of multiple steroidogenic genes (Figure 4.8). Because ASAH1 silencing resulted in a decrease in nuclear S1P levels (Figure 4.5), my findings are not in agreement with Hait *et al.* However, this discrepancy may be due to cell-type specific differences because, in contrast to the nuclear expression of ASAH1 in H295R cells (Figure 5.1), I found that ASAH1 is localized in the cytoplasm of MCF-7 cells (Lucki and Sewer, unpublished observation). Although the present work does not provide direct evidence for the regulation of HDACs and/or histone acetylases by ASAH1, it is tempting to speculate that similar to S1P-mediated inhibition of HDAC activity (255), ASAH1 may play a role in regulating the activity of histone modifying enzymes.

A significant finding of the current work was the effect of ASAH1 silencing on the transcription of members of the NR4A and NR0B subgroups of nuclear receptors (Figure 6.2). ASAH1 is a negative regulator of NR4A genes because suppressing the expression levels of this enzyme resulted in a significant increase in their transcriptional rate (Figure 4.6). Conversely, ASAH1 is a positive regulator of NR0B gene expression (Figure 4.6). Members of the NR4A subfamily of nuclear receptors, namely Nur77, Nurr1, and Nor-1, have recently emerged as key regulators of metabolic functions and are involved in various physiological processes, including apoptosis (532-533), oncogenesis (534), and

steroidogenesis (437,535-538). In steroidogenic cells, Nur77 has been implicated in the regulation of the StAR (536-537), and 3β-HSDII (538) genes. Similarly, Nur77 and Nurr1 activate CYP21A2 (437,535) and CYP11B2 (539) transcription. Because I show that ASAH1 suppression results in increased mRNA and protein expression of Nur77 and Nurr1 (Figure 4.6) concomitant with the upregulation of StAR and CYP21A2 gene expression (Figure 4.2), it is plausible to speculate that at least some of the effects resulting from ASAH1 knockdown are mediated by these nuclear receptors. Of note, Nur77 overexpression was shown to attenuate proliferation of smooth muscle cells (540), which is consistent with the effect of ASAH1 knockdown on the proliferation rate of adrenocortical cells (Figure 4.3).

Similarly, Dax-1 (encoded by NR0B1) is a well-established repressor of SF-1 activity (122,435-436,494). Therefore, lower mRNA transcript levels (and protein expression) of this transcription factor in response to ASAH1 knockdown positively correlate with higher expression of SF-1-regulated genes, including StAR, CYP17A1, CYP11A1, and Mc2R (Figure 4.2). Interestingly, ChIP experiments revealed that ASAH1 is recruited to the DAX-1 proximal promoter in response to $Bt_2$cAMP stimulation (Figure 5.6), suggesting that ASAH1 is a transcriptional regulator of this gene. Therefore, it is possible that the transcriptional regulation of steroidogenic genes by ASAH1 is partially mediated by the modulation of DAX-1 expression (Figure 6.2).

Analogous to Dax-1, SHP (NR0B2) functions as a corepressor for other nuclear receptors (541). SPH is implicated in bile acid synthesis and cholesterol homeostasis (542-544), but a role for this receptor in steroid hormone biosynthesis is yet to be described. Based on the effect of ASAH1 knockdown on SHP gene expression, an intriguing hypothesis of further study is that SHP is a sphingolipid-regulated gene. This may have physiological implications in the liver where SHP plays integral roles in bile acid synthesis and lipid, cholesterol, and glucose metabolism (543). SHP has a

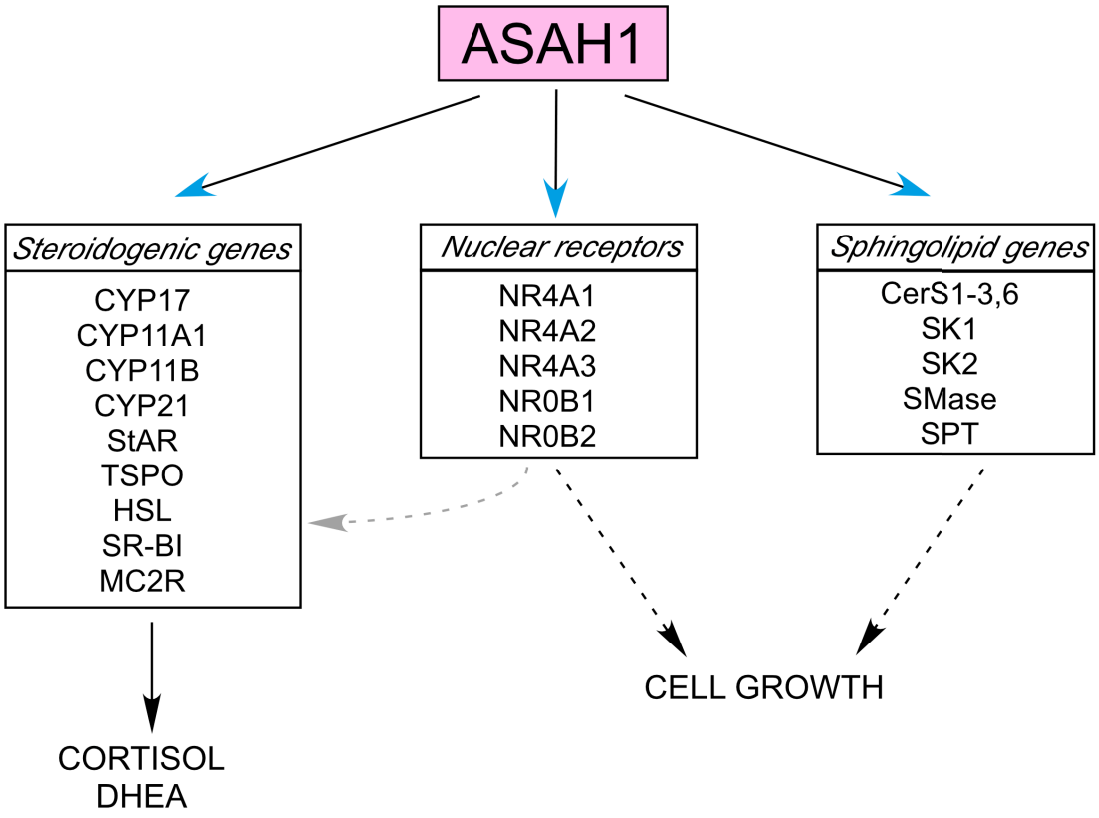



**Figure 6.2.** *ASAH1 is a global regulator of gene transcription in H295R cells.* Schematic representation of the different classes of genes affected by ASAH1 knockdown and their involvement in diverse biological processes, such as steroid hormone production (cortisol and dehydroepiandrosterone, DHEA) and cell growth. Steroidogenic genes encode cholesterol-metabolizing/transport proteins that directly affect steroid hormone production (solid arrow) whereas the protein products of nuclear receptor and sphingolipid genes modulate cell growth indirectly by affecting the expression and/or activity of other genes/enzymes (dashed arrow). NR4A and NR0B genes can also indirectly control cortisol/DHEA synthesis by regulating the transcription of steroidogenic genes (grey dashed arrow).

protective effect in liver fibrosis (545) whereas it promotes dyslipidemia (546) and, consequently, the development of non-alcoholic fatty liver disease (547). Hence, one can imagine a potential role for ASAH1 in the etiology of these conditions. Because SF-1 is a transcriptional activator of both DAX-1 (548-549) and SHP (550), my data indicate that SF-1 requires additional proteins to induce the expression of these genes and that ASAH1 expression (or secondary factors controlled by ASAH1) is essential for the transcription of these genes.

In addition to regulating the expression of steroidogenesis-related genes, ASAH1 expression also affects the sphingolipid metabolic pathway at the transcriptional level (Figure 6.2). Interestingly, my data suggest that ASAH1 is an important factor in regulating overall sphingolipid levels because suppression of ASAH1 resulted in a decrease in the cellular levels of most sphingolipid species quantified, including SPH, S1P, HexCer, and LacCer (Figure 4.5). In nuclei, SM and Cer levels were also decreased in response to ASAH1 suppression (Figure 5.2). Notably, the fold change decrease in sphingolipid levels varied among the different species, with glycosphingolipids (i.e. HexCer and LacCer) being the most affected by ASAH1 knockdown. Transcriptional analysis of sphingolipid genes, for the most part, did not positively correlate with the observed changes in the steady-state intracellular sphingolipid profile of these cells. Nonetheless, it is plausible to consider that ASAH1 may modulate sphingolipid flux by regulating SPTLC2 gene expression (Figure 4.4). The SPT holoenzyme, consisting of SPTLC1 and SPTLC2 subunits, catalyzes the initial and rate-limiting step in *de novo* sphingolipid biosynthesis (Figure 1.2). Although both subunits are essential for catalytic activity (551), SPTLC2 is the only subunit capable of binding the required pyridoxal 5'-phosphate cofactor (552). Therefore, based on the importance of SPTLC2 in forming a catalytically competent enzyme, it is logical that global reduction in sphingolipid levels stems, at least in part, from reduced SPTLC2 mRNA expression in response to ASAH1 silencing. In order to compensate for impaired *de novo* sphingolipid synthesis (as a result of decreased SPTLC2 expression), the cell increases the breakdown of complex sphingolipids (e.g. HexCer and LacCer), thus resulting in a net decrease in the cellular amounts of these lipids. Another unexpected finding was a 20% reduction in whole-cell Cer levels (Figure 4.5) and a 40% decrease in nuclear Cer (Figure 5.2) even though multiple CerS isoforms were transcriptionally induced in response to ASAH1 suppression (Figure 4.4). Interestingly, Mullen *et al.* (553-

554) recently reported that selective knockdown of distinct CerS isoforms results in the accumulation of HexCer, which is in contrast to the levels of this species in ASAH1$^{KD}$ cells. A detailed discussion of this aspect of sphingolipid regulation and the potential physiological implications of perturbed sphingolipid homeostasis is presented later in this Chapter.

### 6.5. ASAH1 as a novel SF-1 coregulator

SF-1 is a master regulator of cell-selective gene transcription within the endocrine system and extensive research has established the roles of this receptor in gene expression, cell differentiation, development, and disease (555-557). Nevertheless, an important question that remains unresolved is: what mechanisms regulate SF-1 function? Multiple post-translational modifications, transcription factors, and coregulators that control receptor activity have been identified. The detection of phospholipids in the receptor LBD (163,167) and findings from our own group demonstrating that PA and SPH are capable of modulating the transactivation potential of SF-1 (47,164) added ligand binding as yet another mechanism of receptor regulation. To this end, the present work provides significant evidence for the role of ASAH1 as a novel coregulator of SF-1 in adrenocortical cells. I show that ASAH1 is a nuclear protein in H295R cells (Figure 5.1) and ACTH/cAMP signaling promotes its recruitment to various steroidogenic gene promoters where it forms a complex with SF-1 (Figure 5.6). Additionally, ASAH1 suppresses the transcriptional activity of SF-1 and disrupts the ability of coactivators to enhance SF-1-dependent target promoter activity (Figure 5.4).

Although SF-1 binds to target DNA as a monomer (147), its activity is potently modulated by its interaction with numerous proteins, including coactivators/corepressors (e.g. p160 family of coactivators, CBP/p300, SMRT, NCoR1) as well as other transcription factors (e.g. NF-κB, SHP, Dax-1, GR, Sp1) and proteins (e.g. β-catenin,

polypyrimidine tract-binding protein-associated splicing factor, p54/NonO) that either induce or repress receptor activity (reviewed in (556)). Generally, coregulators bind to nuclear receptors through LXXLL or LXXLL-related [(L/I)-XX-(I/V)-I and L-XXX-(I/L)-XXX-(I/L)(478)] motifs, where X is any amino acid and L is a leucine (477-478) that can also be substituted for an isoleucine, phenylalanine, or methionine (169). Although the molecular basis of interaction between coactivators or corepressors with nuclear receptors is similar, an important difference between them is that coactivators require the AF-2 core (i.e. LLIEML hexamer domain (118)) for interaction (558-559) while corepressors do not (490-491,560). ASAH1 has multiple putative LXXLL motifs that may mediate its interaction with SF-1. Based on mammalian 2-hybrid studies using truncation mutants of ASAH1, it is likely that multiple functional LXXLL motifs (at least one motifs in each subunit) are involved in SF-1 binding (Figure 5.4B). Accordingly, coIP studies revealed that mutation of an LXXLL motif in the $\alpha$ subunit of ASAH1 prevents its interaction with SF-1 (Figure 5.3F). Significantly, mutation of this motif rendered ASAH1 incapable of suppressing SF-1/$Bt_2$cAMP-dependent CYP17A1 promoter activity (Figure 5.4A), indicating that receptor binding through this motif is essential for ASAH1 function. Of note, our laboratory has previously demonstrated that DGK$\theta$, the enzyme that generates the SF-1 agonist PA, interacts with the receptor through multiple LXXLL motifs (164). Mutation of these motifs attenuates the synergistic activation of CYP17A1 promoter activity, indicating that a direct interaction between DGK$\theta$ and the receptor is important for SF-1-dependent gene transcription (164). Experiments in Chapter Five demonstrated that the same is true for the interaction between ASAH1 and SF-1. Studies aimed at mapping additional interacting regions of ASAH1 and SF-1 are an active area of investigation.

Significantly, in support of a coregulatory role for ASAH1, re-ChIP studies revealed that ASAH1 binds to SF-1 on the proximal promoter of the CYP17A1 and StAR

genes (Figure 5.6), hence modulating receptor function on DNA. Because ASAH1 is also recruited to other SF-1-target genes, such as Mc2R and DAX-1 (Figure 5.6), it is tempting to speculate that a complex between ASAH1 and SF-1 also forms at these promoters. Importantly, the fact that ASAH1 knockdown induces the mRNA expression of these genes (Figures 4.2 and 4.6) is consistent with a repressory function of ASAH1 on SF-1 activity (i.e. ASAH1 suppression results in decreased SPH levels, which leads to increased SF-1 activity and SF-1-dependent gene expression). However, because ASAH1 suppression also altered the transcriptional rate of genes that are not known as SF-1-regulated, such as sphingolipid (Figure 4.4) and NR4A (Figure 4.6) genes, there must be multiple mechanisms (in addition to regulating SF-1 activity) by which ASAH1 affects global gene expression. As will be discussed later in this Chapter, modulation of intracellular sphingolipid levels may be one of these mechanisms.

My data suggest that ASAH1 is functioning as a repressor of SF-1. There are at least three possible ways by which ASAH1 may be inhibiting SF-1 function: (1) ASAH1 suppresses SF-1 function by promoting SPH delivery to the receptor. The fact that ASAH1 was unable to abrogate SF-1-dependent CYP17A1 promoter activity in the presence of the ceramidase inhibitor D-NMAPPD (485) (Figure 5.5) suggests that the catalytic activity of ASAH1 (SPH production) is required for the repressive function of ASAH1. However, further *in vivo* and *in vitro* biophysical analysis of the ligand-binding event is necessary to prove this hypothesis; (2) binding of ASAH1 to the receptor prevents interaction with coactivators. This is illustrated by reporter gene assays where co-expression of ASAH1 and SRC-1 suppresses coactivator-mediated SF-1-induced CYP17A1 promoter activity (Figure 5.4); (3) ASAH1 binding to SF-1 promotes the recruitment of and/or interaction with corepressor complexes that prevent the receptor from inducing transcription. This hypothesis is partially supported by the observation that ASAH1 potentiates the repressory activity of the corepressor SMRT (Figure 5.4). In

agreement, SPH has the same effect on SMRT activity (47). Notably, Dax-1 inhibits SF-1-mediated gene transcription by binding to the receptor through functional LXXLL motifs (493) and acting as an adaptor that recruits other factors, such as the corepressor NCoR, to SF-1 (122). It is tempting to speculate that ASAH1 is functioning in a smilar manner as Dax-1 to inhibit SF-1 activity. Analysis of transcription complex assembly on the CYP17A1 (158) and Mc2R (161) promoters revealed that the formation of SF-1-containing complexes is cyclical and dynamic. Therefore, in order to fully understand the regulatory role of ASAH1 in SF-1-dependent steroidogenic gene transcription, temporal ChIP experiments are necessary to capture the timing and order of ASAH1 recruitment to DNA. ChIP-seq analysis will also be significant to identify ASAH1 binding sites across the genome. Equally important are studies in other cell types to establish ASAH1 function across different tissues. Nonetheless, what can be concluded from my experiments is that ACTH/cAMP signaling promotes the recruitment of ASAH1 and binding to SF-1 at the proximal promoter region of target genes whereby ASAH1 mediates transcriptional repression.

### *6.5.1. Incorporation of our current findings to the model of phospholipid and sphingolipid binding and SF-1 function*

As stated previously, there is compelling evidence for an integral role of ligand binding in regulating the ability of SF-1 to activate target gene expression (163-165,167). However, the mechanistic details of ligand binding as well as how this aspect of SF-1 regulation integrates with post-translational modifications and coregulator binding remains elusive. Even though considerable research is necessary to elucidate the molecular underpinnings of SF-1 regulation, our laboratory is actively investigating the role of ligands in controlling receptor function. To this end, the identification of PA and SPH as ligands for SF-1 (47,164), suggests that ligands are dynamically exchanged

under different cellular conditions. We have shown that $Bt_2$cAMP stimulates nuclear DGKθ activity, which is required for SF1-/ $Bt_2$cAMP-dependent CYP17A1 transcription (164). Accordingly, suppression of DGKθ expression attenuates $Bt_2$cAMP-stimulated CYP17A1 mRNA expression and inhibiting DGKθ activity attenuates SF-1 recruitment to the CYP17A1 promoter (164). Because we have also found that DGKθ directly interacts with SF-1 through multiple LXXLL motifs (164), we postulated that this interaction facilitates PA delivery to the receptor's ligand binding pocket. Conversely, SPH is bound to the receptor under unistimulated conditions and activation of the ACTH/cAMP pathway promotes SPH dissociation from the receptor (47) and possibly promotes PA binding (561). Additionally, SPH antagonizes SRC-1-dependent upregulation of SF-1-mediated CYP17A1 gene expression (47) and disrupts the interaction of SF-1 with SRC-1 and GCN5 (158). To build upon these earlier findings, experiments in this dissertation provide evidence for the integration of ASAH1 into the model of SF-1 transcriptional regulation by ligands.

The current work demonstrates that ACTH/cAMP signaling rapidly activate nuclear ASAH1 activity (Figure 5.2), promotes its recruitment to target gene promoters (Figure 5.6), including CYP17A1, and triggers the formation of a complex with SF-1 on DNA (Figure 5.6). In parallel, ASAH1 expression suppresses basal and SF-1/$Bt_2$cAMP-dependent CYP17A1 promoter activity (Figure 5.3A) as well as abrogates the ability of SRC-1 to upregulate SF-1-dependent CYP17A1 promoter activity (Figure 5.4C). Because SPH attenuates the transcriptional activity of SF-1 and $Bt_2$cAMP stimulation promotes SPH dissociation from the receptor (47), it is counter-intuitive to think that ACTH/cAMP signaling, which activates steroidogenic gene transcription, is promoting an interaction between ASAH1 and SF-1 at target promoters. However, as stated previously, SF-1-containing complex assembly on DNA is dynamic and cyclical

(158,161). In addtion, the potential role of post-translational modifications in modulating ASAH1 activity should also be considered. Tyrosine phosphorylation has been suggested to modulate ASAH1 activity (562-563). Likewise, we have found by mass spectrometry that ASAH1 is basally phosphorylated at multiple residues, including tyrosine 289, which is acutely phosphorylated in response to $Bt_2$cAMP treatment (Lucki *et al.,* unpublished observation). Hence, I postulate that additional studies are necessary to determine the spatio-temporal parameters that govern protein-protein and protein-lipid interactions as well as to fully understand how DGKθ and ASAH1, in response to ACTH/cAMP stimulation, reciprocally control SF-1 activity, and in turn, gene transcription. Dammer *et al.* (158) described the cyclical recruitment of SF-1 and coregulatory complexes to the CYP17A1 promoter in response to $Bt_2$cAMP treatment in adrenocortical cells. SF-1 is maximally enriched on DNA 60 min after stimulation with agonist. SRC-1 and GCN5 recruitment closely follows the kinetics of SF-1 binding (158). In addition, the authors demonstrate that SPH disrupts the cooperativity among SF-1, SRC-1, and GCN5 as well as alters the kinetics of SF-1 DNA recruitment (158), suggesting that SPH suppresses the transcriptional activity of SF-1 not only by inhibiting its recruitment to DNA but also by promoting coactivator complex disassembly.

Based on these findings, I propose a model where activation of the ACTH/cAMP pathway rapidly promotes DGKθ binding and PA delivery to SF-1, thus activating the receptor (i.e. promoter binding and recruitment of coactivator complexes) (Figure 6.3). Shortly after, ASAH1 is recruited to promoter DNA where it forms a complex with SF-1. This interaction facilitates SPH delivery to the receptor, potentially decreasing the receptor's affinity for coactivators and promoting receptor ejection from DNA (Figure 6.3). Interestingly, SPH is an activator of DGK (496-497). Thus, an intriguing hypothesis is that SPH may play a dual role in SF-1 regulation: a direct role by serving as an antagonist for the receptor and an indirect role as an activator of DGK, and thus, PA

production. However, this premise is yet to be tested. Likewise, the kinetic details of protein complex assembly and the temporal parameters that define SF-1 ligand binding is the subject of intense research. Studies centered at this aspect of receptor regulation are being undertaken.

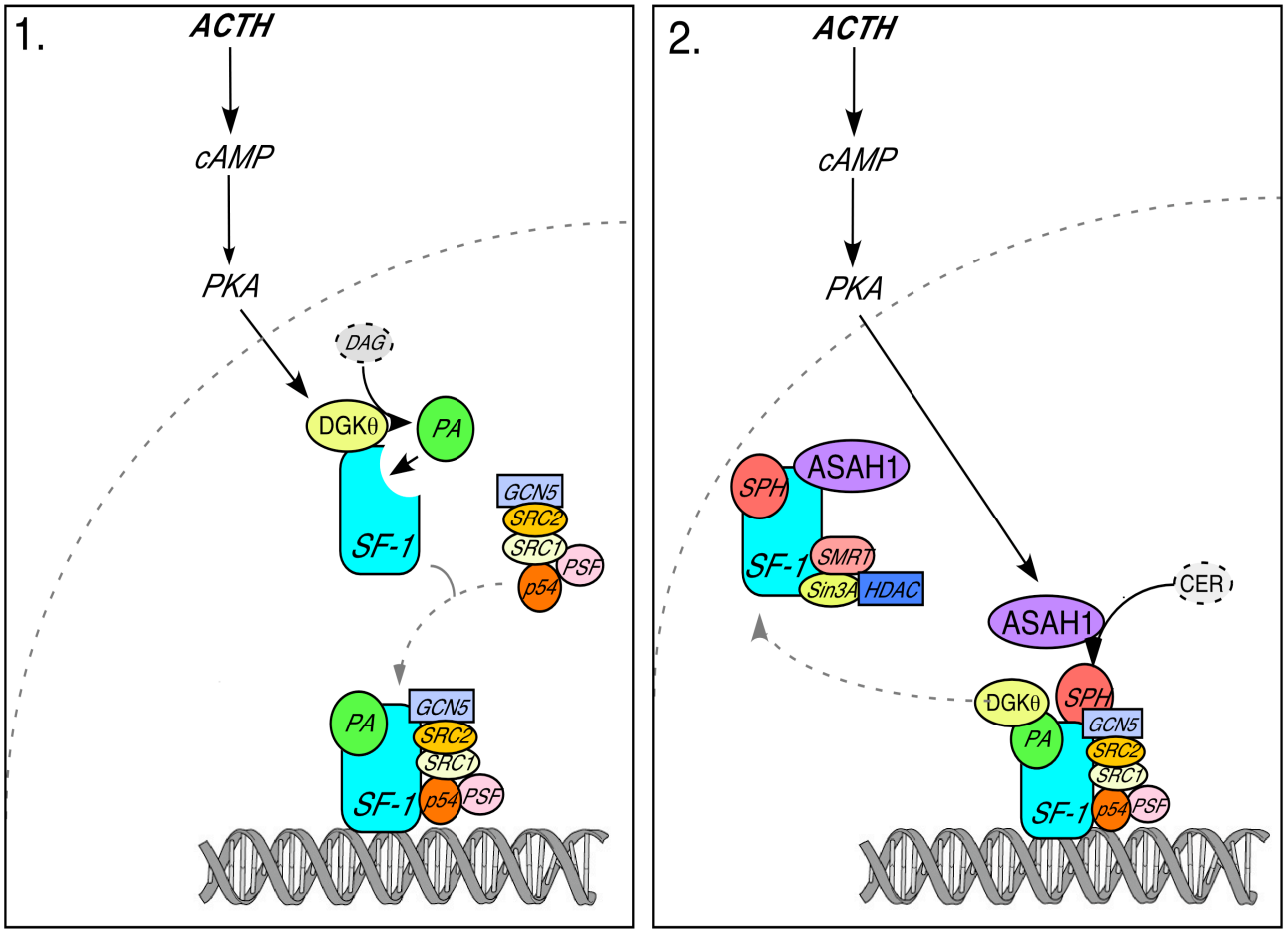



**Figure 6.3.** *Proposed model for the role of ligand binding in regulating SF-1 activity.* (**1**) Protein kinase A (PKA) activation in response to adrenocorticotropin (ACTH) stimulation activates diacylglycerol kinase θ (DGKθ) and promotes phosphatidic acid (PA) binding to SF-1, which activates the receptor. SF-1 binds to DNA, recruits coactivator complexes, and activates transcription. (**2**) After one transcription cycle, ACTH/cAMP signaling promotes ASAH1 recruitment and binding to SF-1 at the promoter of target genes. ASAH1 generates sphingosine (SPH), which disrupts the interaction of SF-1 with coactivators and promotes receptor ejection from DNA as well as its association with corepressor proteins, such as SMRT (silencing mediator for retinoid and thyroid-hormone receptors), HDAC (histone deacetylase), Sin3A. *Abbreviations:* steroid receptor coactivator (SRC), polypyrimidine tract-binding protein-associated splicing factor (PSF), general control nonderepressor 5 (GCN5).

### *6.5.2. Alternative mechanisms of ASAH1 function in adrenocortical cells*

The fact that ASAH1 regulates SF-1 transcriptional activity by directly binding to the receptor (Figure 5.3) raises the premise that this interaction facilitates SPH delivery to the receptor. However, although I provide indirect experimental evidence that supports this hypothesis, as stated previously, specific *in vitro* and *in vivo* biophysical analysis of the ligand delivery and binding events is imperative for proving this theory. Furthermore, the global effects of ASAH1 silencing on gene expression, sphingolipid metabolism, and steroidogenic capacity in adrenocortical cells suggest that ASAH1 may have additional regulatory functions beyond its coregulator role. For example, as discussed in Section 6.4, the fact that ASAH1 regulates the expression of NR4A and NR0B genes, all of which have multiple gene targets, indicates that ASAH1 has the potential to indirectly regulate a wide array of genes in various biological processes. Pleiotropic functions of sphingolipid enzymes are emerging as a common theme in sphingolipid research. For example, SPHK2 not only functions as a secondary coregulator in gene transcription (255) but also promotes cell survival by increasing Cer production through the sphingolipid salvage pathway (190) (Section 1.3).

## 6.6. Implications for the nuclear localization of ASAH1 on its functions

Due to the hydrophobic nature of most sphingolipid species, compartmentalization of sphingolipid metabolism, and subsequently signaling, is an important theme in sphingolipid biology (93). In this manner, Cer localized at the plasma membrane, for example, participates in distinct signaling pathways than mitochondrial Cer (39,554,564-567). Accordingly, induction of apoptosis in rat hepatocytes occurs through the selective accumulation of nuclear Cer due to the activation of ceramidase and neutral SMase at the NE (253). Accumulation of nuclear Cer was also observed in Jurkat T cells in response to Fas ligand-dependent activation of nuclear neutral SMase

and concomitant inhibition of nuclear SM synthase (261). Consequently, the nuclear localization of ASAH1 may be critical for its role as a coregulator of SF-1 as it would promote local production of the receptor's ligand SPH. Because SPH has low water solubility (568), one can envision the binding of ASAH1 to SF-1 as a way to facilitate ligand delivery to the receptor's ligand binding pocket.

Even though all three ceramidase isoforms are expressed in H295R cells (Figure 5.1), several lines of evidence led me to conclude that ACTH/cAMP signaling specifically targets ASAH1 to modulate the steroidogenic metabolic pathway in adrenocortical cells. First, because SF-1 is a nuclear protein, I postulated that the nuclear localization of a ceramidase isoform was imperative. ACER3 is not localized in the nucleus of H295R cells (Figure 5.1A) - and $Bt_2$cAMP stimulation does not promote its nuclear translocation (data not shown)- hence excluding this isoform as a potential candidate for nuclear SPH production and SF-1 regulation. Second, ASAH1 is the only nuclear ceramidase isoform whose activity is upregulated by $Bt_2$cAMP (Figure 5.2). Furthermore, although ASAH2 is also localized in the nucleus of these cells (Figure 5.1), coIP studies revealed that, opposite to ASAH1, ASAH2 not only does not interact with SF-1 (Figure 5.3C), but also is not recruited to the CYP17A1 promoter in respose to $Bt_2$cAMP stimulation (data not shown). Third, ASAH1 knockdown resulted in a decrease in the nuclear levels of SPH and S1P (Figure 5.2). Consistent with a decrease in nuclear SPH levels, ASAH1 suppression led to the transcriptional upregulation of various SF-1 target genes (Figure 4.2).

What can be concluded by the experimental evidence in this dissertation is that, given that SF-1 is a nuclear protein, the nuclear localization of ASAH1 is important for its corepressor role. The nuclear localization of sphingolipid-metabolizing enzymes and the presence of a local nuclear sphingolipid flux is a fairly recent concept. Nonetheless, a growing body of evidence has linked various sphingolipid species to many nuclear

processes (238,444,569). Importantly, some extracellular cues are able to selectively promote nuclear lipid metabolism (570-572). Similarly, some nuclear sphingolipid-metabolizing enzymes have distinct physicochemical properties than their cytosolic counterparts (250), suggesting that the nucleus is a self-regulating sphingolipid metabolic factory. Consequently, the nuclear localization of ASAH1 in adrenocortical cells supports the existence of a local flux of Cer/SPH that may affect diverse nuclear functions, including the transcriptional activity of SF-1. As will be discussed in the next sub-section of this Chapter, the presence of additional nuclear sphingolipid-metabolizing enzymes facilitate a local dynamic flux of many sphingolipid species.

### *6.6.1. Implications for the nuclear localization of Cer/SPH-metabolizing enzymes in ASAH1 function*

The activity of a particular enzyme is not only controlled by extracellular and intracellular factors but is also limited by the availability of substrate. Therefore, given the highly dynamic nature of sphingolipid metabolism, it is plausible to hypothesize that in order for ASAH1 to efficiently produce SPH, Cer-generating enzymes (Figure 1.2) must ensure that the levels of nuclear Cer are optimally maintained. This is accomplished, at least in part, by the nuclear localization of SMase and SM synthase, which together control the balance between SM and Cer. SMase has been detected in the nuclear matrix (248), NE (249), and chromatin (247) while SM synthase has also been identified in the NE and chromatin of various cell types (250). In fact, mass spectrometric analysis revealed that the nuclear levels of SM and Cer are altered in response to $Bt_2$cAMP stimulation (Figure 5.2), thus supporting the nuclear localization of SM- and Cer-metabolizing enzymes in the nucleus of adrenocortical cells. In addition, I found that CerS5 and CerS6 are also localized in the nucleus of H295R cells (Figure 6.4). Hence,

the nuclear localization of multiple SM/Cer-metabolizing enzymes may indirectly influence nuclear ASAH1 function by controlling substrate availability.

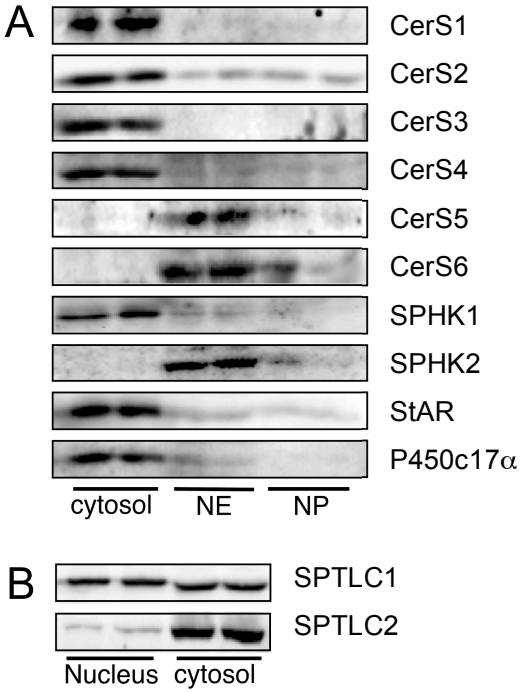



**Figure 6.4.** *Nuclear localization of sphingolipid-metabolizing enzymes in H295R cells.* **A.** Cytosolic, nuclear envelope (NE), and nucleoplasmic (NP) fractions of H295R cells were isolated. Lysates (30 μg of protein) were separated by SDS-PAGE followed by western blotting analysis using antibodies against CerS1-6 (ceramide synthase), SPHK1, and SPHK2 (sphingosine kinase). Mitochondrial StAR (steroidogenic acute regulatory protein) and microsomal P450c17α were used as controls. **B.** Nuclear and cytosolic fractions were isolated from H295R cells and the protein expression of SPTLC1 (serine palmitoyltransferase long-chain subunit 1) and SPTLC2 was detected by western blotting.

The importance of concerted enzymatic action in controlling nuclear sphingolipid function is exemplified by the role of SM on DNA stability during the cell cycle (247). Concomitant activation of SMase and inhibition of SM synthase at the beginning of S phase of the cell cycle leads to a decrease in chromatin-bound SM, which is suggested to facilitate DNA unwinding (236). At the end of S phase, increased SM synthase activity induces an accummulation of chromatin-associated SM, which facilitates double helix restoration after DNA synthesis ends (236). The dynamic balance of nuclear SM gives premise to local Cer metabolism in response to different environmental cues. In fact, as stated above, ACTH/cAMP signaling promotes SM and Cer turnover with a concomitant increase in SPH and S1P levels in the nucleus of H295R cells (Figure 5.2).

In addition to localized Cer production, nuclear SPH metabolism is equally important in controlling its antagonistic action on SF-1 activity. The amount of intracellular SPH is not only regulated by ceramidases but also through its phosphorylation into S1P by SPHKs (Figure 1.3). Nuclear ceramidase activity has been previously identified in rat hepatocytes (252-253). Likewise, nuclear SPHK activity was first described in the NE and nucleoplasm of Swiss 3T3 cells (251). It was subsequenty reported by the same group that SPHK2, as discussed previously, is associated with histone H3 in chromatin of MCF-7 breast cancer cells (255). SPHK2 promotes S1P production and histone acetylation in the nuclei of these cells (255). Of note, SPHK2 is also localized in the nucleus of H295R cells (Figure 6.4). Furthermore, we have found that $Bt_2$cAMP activates SPHK1 (115), and promotes its phosphorylation and nuclear translocation in H295R cells (Li *et al.,* unpublished observations). The nuclear localization of SPHKs in H295R cells supports my mass spectrometric data showing rapid nuclear S1P accumulation in response to $Bt_2$cAMP treatment (Figure 5.2). Interestingly, we have also found that SPHK1 interacts with SF-1 and ASAH1 (Li *et al.,* unpublished observation). This suggests that, in response to ACTH/cAMP signaling, a complex containing SF-1, ASAH1, and SPHK1 may form on promoter DNA, which may be a way to dynamically regulate SF-1 ligand metabolism and HDAC1/2 activity.

The functional implications for the nuclear localization of additional sphingolipid-metabolizing enzymes on the role of SPH as an antagonist of SF-1 are two-fold. First, the nuclear localization of SM/Cer-metabolizing enzymes indicates the presence of a nuclear SM cycle where a dynamic balance between SM and Cer exist. This suggests that, in addition to modulating nuclear ASAH1 activity, ACTH/cAMP signaling may also regulate the activity of SM synthase, SMases, and CerS to maintain optimal nuclear Cer availability for ASAH1 action. Of note, nuclear SM levels are decreased in response to stimulation with $Bt_2$cAMP in WT cells but no change in nuclear SM concentrations were

seen in ASAH1$^{KD}$ cells under the same conditions (Figure 5.2). Second, the signaling properties of newly generated SPH are controlled by nuclear SPHKs, which would limit the antagonistic function of SPH. As discussed above, nuclear SPHK2 and SPHK1 may participate in SPH metabolism in H295R cells. Thus, it is evident that dynamic nuclear sphingolipid metabolism plays an integral role in facilitating SF-1 ligand exchange.

### 6.7. Implications of the current work to the field of sphingolipid research

It has become very clear that sphingolipids are essential for physiological homeostasis and, as such, intricate networks are involved in their regulation. Accordingly, understanding the mechanistic underpinnings of sphingolipid metabolism as well as the effects of sphingolipid dysregulation on overall cellular function is imperative. To this end, mice knockout models of different sphingolipid enzymes, including CERK (573), GM3 synthase (574), SPHKs (575), ASAH2 (576), SPL (577), and CerS2 (578), have been generated. However, because disruption of ASAH1 expression is embryonically lethal (109), a global knockout model for this enzyme is not feasible. The effect of ASAH1 knockout not only exemplifies the essential role of this enzyme in development but also indicates that the other ceramidase isoforms cannot compensate for loss of ASAH1. It is likely that future studies using tissue-specific or conditional knockout mouse models may provide new insights into ASAH1 function. The significance of the findings presented in this dissertation to the field of sphingolipid research is multifold. First, a novel regulatory role for S1P in adrenocortical steroid hormone synthesis was established. Second, I functionally characterized the human ASAH1 promoter and established the ACTH/cAMP pathway as an activator of ASAH1 expression at both transcriptional and post-transcriptional levels. Third, a novel role for ASAH1 as a global regulator of gene expression was uncovered. Finally, I identified ASAH1 as a nuclear protein in H295R cells and defined its function as an SF-1

coregulator. To my knowledge, this is the first demonstration that a sphingolipid-metabolizing enzyme serves such function. Although the current work focuses on adrenocortical steroid hormone production, my findings have global implications as it highlights the importance of sphingolipids in the maintenance of hormones that are required for varied physiological processes (Section 1.1). Furthermore, characterization of the ASAH1$^{KD}$ cell line provides valuable information about the contribution of ASAH1 to the steady-state levels of sphingolipids.

### *6.7.1. Potential role of ASAH1 in the maintenance of sphingolipid homeostasis*

Mass spectrometric analysis of sphingolipid content in response to ASAH1 suppression revealed interesting yet unexpected results that implicate ASAH1 as an important enzyme not only for the regulation of Cer/SPH/S1P levels but also for the maintenance of overall sphingolipid homeostasis. The most stricking consequence of ASAH1 silencing was a decrease in the steady-state levels of most sphingolipid species quantified (Figure 4.5 and 5.2). A commonality among published studies of knockout (or knockdown) models of sphingolipid-metabolizing enzymes is the role of compensatory mechanisms to sustain homeostatic conditions. Likewise, these studies highlight the fact that suppression of one enzyme can affect cellular sphingolipid composition in unpredicted ways. For example, downregulation of CerS2 in neuroblastomal cells increased the levels of long-chain Cer and SM, partially due to the upregulation of ACER1 and 2 reverse activities (579). Similarly, selective knockdown of different CerS isoforms in MCF-7 breast cancer cells revealed complex transcriptional inter-regulation among these enzymes, but the changes in sphingolipid content were not readily accounted for by the changes in mRNA expression of the different enzyme isoforms (553). Accordingly, my findings suggest that ASAH1 silencing have significant effects on

the sphingolipid profile of adrenocortical cells. These changes were thoroughly discussed in Section 4.4 of Chapter Four.

Several recent studies have provided insights into the molecular mechanisms by which cells sense and coordinate sphingolipid production. As stated above, the overall decrease in sphingolipid content in response to ASAH1 silencing supports a role for ASAH1 in maintaining optimal steady-state sphingolipid levels. The outcome of ASAH1 suppression on cellular sphingolipid content can be partially due to suppression of SPTLC2 expression (Figure 4.4 and Section 6.4). In addition, because sphingolipid dysregulation is known to cause cellular stress and pathological effects (580-582), it is significant that ASAH1 knockdown resulted in reduced cellular proliferation, with no change in cell viability (data not shown), than WT cells (Figure 4.3). This effect can be partially explained by a decrease in the expression levels of cyclin B2 (Figure 4.3), which is expressed during G2/M phase of the cell cycle and regulates mitotic progression (583), and PCNA (Figure 4.3), which is essential for DNA replication by serving as a sliding clamp for DNA polymerase (584). It is possible that ASAH1 indirectly modulates the function of additional proteins that maintain optimal sphingolipid levels and/or control cell growth.

To date, three groups of proteins have been implicated in the regulation of steady-state sphingolipid levels: TORC2 (target of rapamycin complex 2), SMSr (SM synthase-related), and ORMs. TORC2 is comprised of TOR (target of rapamycin), rapamycin-insensitive companion of TOR, GβL, and stress-activated protein kinase interacting protein 1 (585). In mammalian cells, this complex has been implicated in the regulation of actin cytoskeleton and cell survival (585). TORC2 kinase complex activity was shown to be required for maximal CerS activity (586-587), thus for normal levels of sphingoid bases (e.g. SPH and sphinganine, Figure 1.3), sphingoid base phosphates (e.g. S1P), and Cer. Similarly, SMRr, a Cer ethanolamine phosphotransferase that

synthesizes Cer phosphoethanolamine (588), was shown to act as a Cer sensor and modulate sphingolipid production in response to Cer levels (588). Suppression of SMSr causes a dramatic increase in Cer and glucosylceramide levels (588), suggesting that when this sensor is blocked, unregulated Cer accumulation occurs. Both TORC2 and SMSr activity are necessary for normal sphingolipid metabolism, although the intricate details of how they act upon the sphingolipid pathway are yet to be detailed. More recently, ORM proteins identified in yeast (ORMDL in humans (589)) were implicated in a mechanism by which cells can adjust sphingolipid content according to metabolic demand (580,590). ORMs are ER-localized transmembrane proteins with no known functional domains. However, they are strongly conserved across species (580), suggesting that they share a common function. ORM overexpression reduces sphingolipid concentrations (591) whereas constitutive loss of ORM gene function causes toxic overproduction of sphingolipids (580), which suggest that these proteins are negative regulator of sphingolipid synthesis. Although the present work did not include an in depth analysis of possible regulatory pathways affected by ASAH1 silencing, based on these recent discoveries of sphingolipid-regulating proteins, it is provocative to speculate that ASAH1 may play a role in controlling the function of these proteins.

### *6.7.2. Potential implications for altered steady-state sphingolipid levels resulting from ASAH1 suppression*

SM and glycosphingolipids (e.g. HexCer and LacCer) are integral components of cell membranes, along with glycerolipids and sterols (592). The complex head-group glycans of glycosphingolipids serve as cell surface-exposed membrane elements that can be recognized and bound by a series of proteins (592). Therefore, the most obvious implication of altered cellular sphingolipid composition in adrenocortical cells is changes

in the biophysical properties of lipid bilayers. Because of the physical and functional relationships among sphingolipids, glycerolipids, and sterols, perturbations to sphingolipid metabolism may affect the amount and location of these other lipid classes. Finally, it is now well established that signal transduction initiated at the cell membrane is influenced by lipid-microenvironments, known as lipid rafts, which are comprised of sphingolipids and cholesterol and serve as organizing centers for the assembly of signaling molecules (593-594). Therefore, alterations in intracellular sphingolipid content may also affect the formation of these membrane domains, thus impairing cellular responses to extracellular cues.

Cholesterol is not only important for membrane fluidity but also, as it pertains to the present work, is the precursor for steroid hormones (Section 1.1). Extensive evidence has established the coregulation between sphingolipids and cholesterol. Their extracellular trafficking occurs through the same lipoprotein particles (595) and mutations of either sphingolipid- or sterol-metabolizing enzymes cause accumulation of both lipid types (577,596). Furthermore, decreases in membrane sphingolipid levels promote the redistribution of plasma membrane cholesterol into intracellular membranes (e.g. ER), which represses the activity of SREBP2, the major transcriptional regulator of cholesterol uptake and biosynthesis (597-599). Conversely, treatment of cells with exogenous sphingolipids promotes the accumulation of cholesterol at the plasma membrane and a drop in ER-localized cholesterol, which induces the activation of SREBP2 and *de novo* cholesterol synthesis (506,600). Given that suppression of ASAH1 expression resulted in decreased levels of HexCer and LacCer (Figure 4.5), one can envision a role for ASAH1 in maintaining optimal membrane fluidity and, to a broader extent, regulating plasma membrane-associated cholesterol levels.

Glycerolipids have many biological functions and perturbations to their metabolism lead to a range of pathophysiological conditions, including obesity, type II

diabetes, non-alcoholic fatty liver disease, and cancer (reviewed in (601)). Sphingolipids and glycerolipids share many direct metabolic connections. As such, it is plausible to infer that changes in cellular sphingolipid content may alter glycerolipid metabolism. For example, the transfer of a phosphorylcholine group from phosphatidylcholine to Cer forms SM, thus generating DAG as a bi-product (Figure 1.3). In addition, phosphatidylinositol provides the source of phosphoinositol for the generation of inositolphosphorylceramide in yeast (602). Conversely, the degradation of S1P forms ethanolamine phosphate, which can be recycled for use in phosphatidylethanolamine (PE) synthesis. Interestingly, studies in *Drosophila* have found that sphingolipids (e.g. long-chain sphingoid bases and Cer) are able to regulate the SREBP pathway, which senses and regulates PE synthesis (rather than cholesterol) in this organism (603). These examples illustrate the ability of sphingolipids to support glycerolipid synthesis and the interconnection between the metabolisms of these two lipid groups. Hence, ASAH1-dependent dysregulation of steady-state sphingolipid levels may affect glycerolipid-regulated cellular functions.

The importance of sphingolipids in cellular processes is underscored by the role of lipid rafts (i.e. detergent-resistant domains) in membrane fluidity, membrane protein trafficking, and intracellular signaling (593,604). These membrane microdomains are formed by SM, glycosphingolipids, cholesterol, and lipid-modified proteins (e.g. caveolins, Src-family of kinases, flotillins), which together are proposed to spacially organize signaling molecules to promote kinetically favorable connections for signaling transduction (605). For example, a negative correlation between membrane-associated glycosphingolipid concentrations and insulin responsiveness exists in a way that excessive glycosphingolipid content hampers insulin receptor signaling (606). Similarly, lipid raft-associated LacCer is important for phagocytosis, chemotaxis, and superoxide generation in neutrophils (607). Importantly, small changes in the amounts or structure

of membrane-embedded lipid molecules are known to greatly influence the behavior of lipid rafts (608-609). It was recently reported that combined knockdown of CerS5 and CerS6 results in a decrease in long-chain Cer levels and plasma membrane permeabilization (554). Therefore, it is possible that decreased HexCer and LacCer concentrations in response to ASAH1 knockdown may affect lipid raft formation and thus impair cellular signaling. Furthermore, the fact that nuclear SM and Cer levels were decreased in ASAH1$^{KD}$ cells (Figure 5.2) suggests that nuclear membrane composition and biophysical properties are also affected by changes in ASAH1 expression.

### 6.8. Implications of the current work to health and disease

The studies described in this dissertation characterized the functions of ASAH1 in ACTH/cAMP-dependent steroid hormone production in adrenocortical cells and established this ceramidase as an important factor for steroidogenic homeostasis. Because abnormal cortisol and adrenal androgen levels occur in several pathophysiological states, including Cushing's syndrome, polycystic ovary syndrome (PCOS), and cancer (reviewed in (610-613)), the present work has several health implications. Given the role of S1P as an inducer of cortisol production (Chapter Two) and the importance of ASAH1 expression in maintaining optimal adrenocortical steroidogenic capacity (Chapter Four), it is plausible to theorize that aberrant ASAH1 expression may be involved in the etiology of these pathophysiological conditions. Concurrently, because ASAH1 is a negative regulator of NR4A nuclear receptor gene expression (Figure 4.6) and these receptors are linked to many metabolic disorders (e.g. insulin resistance and dyslipidemia), potential implications for ASAH1 in these conditions are also possible.

### *6.9.1. Glucocorticoids and Cushing's syndrome*

Cushing's syndrome is characterized by the hypersecretion of cortisol (614-615). Due to the pleiotropic functions of cortisol (e.g. gluconeogenesis, lipolysis, hyperconstriction, and suppression of the innate inflammatory response (616-619)), this syndrome has a vast array of systemic effects. The most prevalent causes of endogenous Cushing's syndrome are an ACTH-secreting pituitary adenoma (i.e. Cushing's disease) and adrenocortical hyperplasia, adenoma, or carcinoma, where cortisol is hypersecreted from the tumor independently of ACTH stimulation (614). Primary Cushing's syndrome is most often secondary to adrenocortical tumors. However, adrenocortical cancer is a rare disease (615) and as such, little is known about its etiology. Because ASAH1 is a regulator of cortisol production (Chapter Four), it is tempting to speculate that abnormal ASAH1 expression may be characteristic of adrenocortical tumors. Although this ceramidase isoform is overexpressed in many cancer types (110-113), its expression level in adrenocortical cancer is yet to be reported.

Due to the opposing roles of Cer and S1P in cell survival (i.e. Cer induces apoptosis while S1P is a potent mitogen (45,197)), the intracellular balance between these two metabolites, which is controlled by ceramidases, could be an underlying factor in adrenocortical tumorigenesis, and subsequently, Cushing's syndrome. As discussed previously, the nuclear localization of ASAH1 in adrenocortical cells infers that this ceramidase is important for controlling the intranuclear concentrations of Cer and S1P. In cancer, this regulation is important because nuclear Cer and S1P have both been implicated in the transcriptional regulation of cancer-related genes through epigenetic mechanisms (255,620). SPHK2-generated nuclear S1P can bind to the active site of HDAC1/2, inhibit their activity, and consequently induce C-FOS transcription (255). Conversely, Cer inhibits the mRNA expression of the pro-metastatic enzyme MMP-2

(matrix metalloproteinase 2) by promoting a decrease in histone acetylation at the MMP-2 promoter (620). Lastly, S1P is a well-known oncogenic agent that promotes tumor survival, growth, and metastasis (185,621-622). Hence, this metabolite may be a contributing factor for the onset of Cushing's syndrome not only by activating cancer-promoting signaling pathways in adrenocortical tumors but also by directly activating cholesterol-metabolizing pathways and promoting cortisol production (Chapter Two).

Interestingly, many of the deleterious clinical manifestations associated with Cushing's syndrome, such as insulin resistance and hypertension (623), have been linked to aberrant sphingolipid metabolism. Cer antagonizes insulin signaling by inhibiting the membrane translocation (624) as well as the phosphorylation and activation of Akt (625), the obligate intermediate in the insulin signaling pathway. Cer has also been proposed to impair insulin action by inducing the formation of reactive oxygen species (626-627), which promote insulin resistance, and mediates TNF-$\alpha$-induced downregulation of the insulin-responsive glucose transporter (GLUT4) in adipocytes (628). Glucocorticoids, which are known to induce insulin resistance (629), can influence insulin action by modulating the sphingolipid pathway. Cer has been described as an intermediate linking glucocorticoids to the induction of insulin resistance. Dexamethasone promotes Cer accumulation by inducing the expression of various sphingolipid-metabolizing enzymes including SPTLC2 and CerS (527). Furthermore, dexamethasone antagonizes insulin-stimulated glucose uptake by promoting an increase in membrane SM levels (630) whereas adrenalectomy not only increases insulin sensitivity but also significantly decreases adipocyte SM levels (631). Similarly, sphingolipids may contribute to hypertension by altering membrane fluidity (609) as well as inhibiting nitric oxide accumulation, which has strong vasodilatory functions (632). Collectively, these findings give premise to a mutual inter-regulation between glucocorticoids and sphingolipids where sphingolipids control glucocorticoid synthesis in

the adrenal cortex while glucocorticoids influence sphingolipid metabolism at numerous target tissues. As it relates to the present work, this suggests that aberrant ASAH1 espression and/or S1P levels that lead to excessive cortisol production have systemic pleiotropic effects that may be related to the pathogenesis of Cushing's syndrome. Future studies using mouse models with conditional deletion of ASAH1 in the adrenal gland may provide insight into the role of this ceramidase in modulating glucocorticoid-dependent physiological responses.

### *6.9.2. Adrenal androgens and polycystic ovary syndrome*

PCOS is a heterogeneous disorder characterized by chronic anovulation and aberrant androgen production (633). Although the ovaries are the main source of increased androgens in this condition, elevated levels of adrenal androgens, mainly DHEA-sulfate (DHEAS), is observed in about 40% of patients (634). DHEAS, produced in the zona reticularis of the adrenal cortex (Section 1.1), is the second most abundant steroid, after cortisol, and is converted into testosterone and estrogens (e.g. estrone, estradiol, and estriol) in peripheral tissues (635). Therefore, plasma DHEAS is a marker for adrenal androgen secretion and a diagnostic factor for PCOS. Adrenal androgen excess correlates with an exaggerated secretion of DHEAS from the adrenal cortex rather than altered pituitary responsiveness to chorticotropin-releasing hormone or increased adrenocortical ACTH responsiveness (636-637). Given that ASAH1 is a negative regulator of DHEA secretion (Chapter Four) and steroidogenic gene transcription (Chapters Four and Five) in adrenocortical cells, aberrant ASAH1 expression may be a contributing factor for the manifestation of PCOS.

The causes for adrenal hyperandrogenism are not fully understood, but researchers have shown that the activities of P450c17$\alpha$ and 3$\beta$-HSDII enzymes, both of which are essential for androgen synthesis (Section 1.1), are upregulated in PCOS

samples (638-639). In addition, increased steroid hormone secretion in PCOS ovarian theca cells is associated with the transcriptional upregulation of various steroidogenic genes including CYP17A1 (640-641), CYP11A1 (640), 3β-HSDII (638), and StAR (640). Although it is logical that not one single mechanism is responsible for the complex changes in gene expression associated with the PCOS phenotype, based on the global changes in gene transcription resulting from ASAH1 knockdown (Chapter Four), it is provocative to speculate that aberrant sphingolipid metabolism, and more precisely, ASAH1 expression, may be a causal factor for disregulated steroidogenic gene transcription in this syndrome.

Similar to Cushing's syndrome, PCOS is also associated with the development of insulin resistance (642). Although the mechanism of impared glucose utilization in PCOS is largely unknown, a link between androgens and insulin resistance has been proposed. Increased insulin sensitivity was observed during androgen suppression (643) or with anti-androgen treatment (644). Conversely, studies have suggested that hyperandrogenism results from hyperinsulinemia as insulin and IGF-I stimulate steroidogenesis from ovarian (645-647) and adrenal (648-650) tissues. Of note, PCOS is also associated with dyslipidemia (546), a condition where, as discussed previously, the nuclear receptor SHP plays a prominent role. SHP is involved in multiple aspects of lipogenesis (543) including the transcriptional regulation of genes associated with lipid metabolism, such as PPARγ (651), SREBP-1c (652), and microsomal triglyceride transfer protein (653). Furthermore, SHP knockout mice are protected against dyslipidemia (546) and have increased adipocyte energy utilization (654). Therefore, even though a direct link between PCOS and sphingolipids is yet to be established, based on the role of ASAH1 as a global regulator of gene expression, including SHP (Figure 4.6), and adrenocortical steroidogenic capacity (Figure 4.3), it is logical to think

that ASAH1 is probably involved in the onset of secondary pathophysiological states associated with PCOS.

### *6.9.3. NR4A nuclear receptors and energy metabolism*

The prominent effect of ASAH1 suppression on the mRNA and protein expression of members of the NR4A subfamily of nuclear receptors (i.e. Nur77, Nurr1, and Nor-1) (Figure 4.6), implicate ASAH1 to various NR4A-regulated cellular processes. Emerging evidence has established members of the NR4A subgroup as key transcriptional regulators of lipid, carbohydrate, and energy homeostasis (reviewed in (655)). Hence, these receptors play roles in the development of various pathophysiological conditions including cardiovascular disease (656), inflammation (657), and metabolic diseases (658). Of note, because endogenous ligands for these receptors have not yet been identified, their activity may be regulated by expression, or perhaps by a yet to be identified lipid.

NR4A receptors are linked to multiple metabolic functions. In skeletal muscle, NR4A expression is regulated by β-adrenergic signaling, an integral regulator of metabolic homeostasis (659). Nur77 regulates the transcription of genes involved in glucose transport, insulin signaling, and glycolysis (655) while Nor-1 control genes involved in oxidative metabolism (660). Accordingly, Nor-1 knockdown results in decreased fatty acid oxidation and greater lactate production (660) while loss of Nur77 leads to impaired insuling signaling (661). In the liver, NR4A genes are induced by glucagon and fasting (662), and all three receptor subtypes induce hepatocyte glucose production (662). In adipose tissue, Nur77 overexpression inhibits adipogenesis (663-664) whereas Nur77 knockout prevents β-adrenoreceptor-mediated thermogenesis (665).

Interestingly, NR4A receptors have also been implicated in dyslipidemia and insulin resistance, both of which, as stated above, are linked to Cushing's syndrome and PCOS. NR4A receptors control the expression of numerous genes involved in lipid metabolism and insulin signaling. Nur77 regulates lipid metabolism by acting as a negative regulator of SREBP-1c (666) and glucose-6-phosphatase (662) expression while being an inducer of fatty acid translocase (Cd36) transcription (667). Similarly, Nor-1 controls the expression of PGC-1$\alpha$ and lipin1 (660,668). As it relates to insulin function, Nur77 modulates insuling signaling by controlling GLUT4 and AMP-activated protein kinase gene transcription (667). In adipocytes, insulin induces Nor-1 and Nur77 expression (658) and Nor-1 overexpression increases insulin-stimulated Akt phosphorylation, GLUT4 plasma membrane translocation, and glucose uptake (658). These findings not only suggest that NR4A receptors play a role in some of the etiologies of Cushing's syndrome and PCOS but also lend support to the potential role of ASAH1 in the regulation of energy homeostasis and associated pathophysiological conditions.

### 6.10. Concluding remarks

Adrenocortical steroid hormones are essential for physiological homeostasis. As such, intricate molecular mechanisms control their biosynthesis. This dissertation was aimed at elucidating the role of sphingolipids in ACTH-dependent steroidogenesis by defining the role of S1P in acute cortisol biosynthesis as well as defining the functions of ASAH1 in multiple aspects of steroidogenic regulation in the human adrenal cortex. Completion of these studies led to the following conclusions: (1) S1P is an inducer of acute cortisol production in adrenocortical cells, (2) CREB is an essential transcriptional activator of the ASAH1 gene, (3) ASAH1 is a global regulator of steroidogenic gene

transcription and steroidogenic capacity in the adrenal gland, and (4) ASAH1 is a novel SF-1 coregulator.

## APPENDIX 1:

*Genistein induces MCF-7 breast cancer cell growth by inducing acid ceramidase gene expression*

### A1.1. Introduction

Genistein (4,5,7-trihydroxyisoflavone) is an isoflavone that is abundantly present in soybeans, vegetables, and fruits (669). It is classified as a phytoestrogen because it has estrogenic effects in animals. In a broad sense, phytoestrogens are capable of binding to ERs, thereby modulating ER signaling pathways (670). Due to their estrogenic properties, these plant-based compounds have attracted significant research interest and are considered, for the most part, natural chemopreventive agents (671-672). Genistein is the predominant isoflavone in soy products (673), and therefore, has been the subject of much research, mainly focused on its effects on the growth of $E_2$-dependent mammary tumors both *in vitro* and *in vivo* (reviewed in (674)). Functional studies determined that genistein has properties of a selective ER modulator. In this manner, genistein activates numerous $E_2$-dependent signaling cascades and induce gene transcription by modulating the activity of ERs (675-676). Binding studies revealed that, even though genistein competes with $E_2$ for binding to ER$\alpha$ and ER$\beta$, it does so with different affinities. The binding affinity of genistein for ER$\alpha$ and ER$\beta$ is 4% and 87%, respectively, compared to $E_2$ (677-679). Furthermore, genistein also activates GPR30 (680-682), a GPCR that binds most ER ligands and mediates rapid estrogenic signaling (683-684).

Genistein exerts many cellular effects including activating apoptosis, inhibiting protein tyrosine kinase activity, and suppressing angiogenesis (685-686). Genistein also acts in a dose-dependent manner to both positively (687-691) and negatively (678,692-697) regulate tumorigenesis. In ER-positive cells, high doses of genistein (> 10 $\mu$M) are associated with tumor suppression while low doses (0.01 - 1 $\mu$M) have a mitogenic effect

(693,698-699). Genistein promotes proliferation of $E_2$-dependent breast and thyroid cancer cells (680,694,700), and induces the expression of vascular endothelial growth factor (701), C-FOS (681), PPAR$\gamma$ (702), and proteinase inhibitor 9 (703). The proliferative properties of genistein are mostly due to its ability to activate genomic and non-genomic estrogenic pathways by binding to ER$\alpha$, ER$\beta$, and GPR30 (695). The binding of $E_2$ to GPR30 stimulates the cAMP pathway (704), increases intracellular $Ca^{2+}$ (683-684), and induces epidermal growth factor receptor transactivation in ER-negative breast cancer cells (705-706). Signaling through GPR30 promotes the proliferation of multiple carcinomas (707-708), stimulates cell migration through induction of the connective tissue growth factor gene (709), and promotes C-FOS (681), Bcl-2 (710-711), cyclin D2 (712), and estrogen-related receptor $\alpha$ (713) gene expression.

Sphingolipids are involved in many aspects of cell regulation (reviewed in (89,440,443)). Cer and S1P have been extensively studied for their opposing roles in the regulation of various aspects of cancer pathogenesis and therapy (714). Cer inhibits cell growth and promotes apoptosis and senescence, while S1P induces cell proliferation and migration by signaling through five S1PRs (44,82,106) (Figure 1.8). In this manner, the relative concentrations of these two molecules determine if the cell undergoes apoptosis or proliferates (714). Consequently, targeting pathways that elevate Cer or decrease S1P accumulation has been a promising therapeutic approach in cancer treatment (56).

ASAH1 regulates Cer metabolism by catalyzing its degradation to SPH and a free fatty acid (423). SPH is phosphorylated by SPHKs to form S1P. Because Cer hydrolysis is the major pathway for SPH generation (715), ASAH1 plays a key role in regulating cellular homeostasis by controlling the Cer/SPH/S1P balance within the cell. In addition, the aberrant expression of ASAH1 in various human cancers (110-113), including breast cancer (716), prompted the emergence of this enzyme as a potential

target for chemotherapy (112,717-718). Inhibitors of ASAH1 such as B13 [(1R,2R)-2-(N-tetradecanoylamino)-1-(4-nitrophenyl-1,3-propanediol)] and LCL464 (an analog of B13) were shown to cause Cer accumulation and prevent tumor growth (717,719). At the transcriptional level, functional characterization of ASAH1 has been previously reported (423-424), and I (Chapter Three) and others (425) have established that CREB and KLF6 are important transcriptional regulators of this gene.

Despite the prominent roles of sphingolipids in cancer development and the proliferative actions of low-doses of genistein, little is known about the relationship between these two factors in cancer progression. Therefore, based on the importance of ASAH1 in sphingolipid metabolism, the role of Cer/S1P balance in carcinogenesis, and the numerous biological effects of genistein in cancer cells, I sought to determine the role of genistein in ASAH1 gene transcription in MCF-7 breast cancer cells. In this Chapter I show that genistein induces ASAH1 gene expression through an ERK1/2-dependent mechanism involving both GPR30 and ER$\alpha$. Furthermore, I demonstrate that ASAH1 expression is required for genistein-stimulated cyclin B2 expression and MCF-7 cell proliferation.

## A1.2. Materials and Methods

### *A1.2.1. Cell Culture*

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in phenol red-free Eagle's minimum essential medium (MEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech, Inc.), 11 mM sodium pyruvate (Sigma), 0.01 mg/ml bovine insulin (Sigma), antibiotics, and antimycotics. MDA-MB-231 human breast cancer cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc.) supplemented with 10% bovine calf serum (Mediatech, Inc.), antibiotics, and antimycotics.

### *A1.2.2. Real Time RT-PCR (qRT-PCR)*

MCF-7 cells were sub-cultured into 12-well plates and 48 h later treated with 20 nM genistein or 10 nM G-1 for 1 - 24 h. In some experiments, cells were pre-incubated with 100 - 200 nM G-15 for 1 h prior to treatment with genistein or G-1. Total RNA was isolated and quantified by qRT-PCR as previously described (461). ASAH expression was normalized to β-actin mRNA levels and calculated using the delta-delta cycle threshold (ΔΔCT) method.

### *A1.2.3. RNA interference (RNAi) and real time RT-PCR*

MCF-7 cells were sub-cultured into 12-well plates and 24 h later transfected with 75 nM non-specific, 100 nM GPR30 (L-005563-00-0005, Dharmacon/Thermo Scientific), or 75 nM ERα (L-003401-00-0005, Dharmacon/Thermo Scientific) small interfering RNA (siRNA) oligonucleotides using HiPerfect Transfection Reagent (Qiagen, Valencia, CA). Twenty-four h later, cells were transfected again, incubated for an additional 24 h, and then treated with 20 nM genistein or 10 nM G-1 for 24 h. Total RNA was isolated and

quantified by qRT-PCR as described above. Reduction of GPR30 and ERα protein expression at the time of RNA isolation was confirmed by western blotting.

### *A1.2.4. ERα transient transfection*

MDA-MB-231 cells were sub-cultured into 12-well plates and transfected with 1 μg pCMV-hERα (kindly provided by Dr. Ann Nardulli, University of Illinois, Urbana, IL) using GeneJuice (EMD Biosciences). Twenty-four h after transfection, cells were treated with 20 nM genistein or 5 nM $E_2$ for 24 h. Total RNA was isolated and ASAH1, pS2, and β-actin mRNA levels were quantified by qRT-PCR as described above. Expression of ERα was confirmed by western blotting 48 h after transfection.

### *A1.2.5. Western blotting*

For quantification of ASAH1 protein expression, MCF-7 cells were treated with 20 nM genistein for 24, 48, or 72 h and harvested into RIPA buffer. Cell lysates were isolated and separated by SDS-PAGE as previously described (461). Western blots were incubated with anti-ASAH1 (HPA005468, Sigma) and anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. For quantification of ERK1/2 activation, MCF-7 cells were serum-starved for 40 h, then treated with 20 nM genistein for 0 to 30 min. ERK1/2 phosphorylation was determined by western blotting using an anti-phospho-ERK1/2 antibody (sc-7383, Santa Cruz) and normalized to total ERK2 expression (sc-154, Santa Cruz). For quantification of phosphorylated ERα, MCF-7 cells were serum-starved for 40 h, then treated with 20 nM genistein for 10, 30, or 60 min. ERα phosphorylation at serine-118 was determined by western blotting using an anti-phospho-$Ser^{118}$-ERα antibody (clone NL-44, Millipore) and normalized to total ERα expression (H-184, Santa Cruz). For quantification of cyclin B2 protein expression, MCF-

7 cells were serum-starved for 48 h and then pre-treated with 200 ng/mL nocodazole (Calbiochem Inc.) for 10 h. Cells were rinsed 3 times with PBS, serum-free MEM medium was added to each well, and cells were treated with 20 nM genistein for 6 or 12 h. In some experiments, cells were transfected with 75 nM ASAH1 siRNA oligonucleotides (M-005228-01-0005, Dharmacon/Thermo Scientific) using HiPerfect Transfection Reagent (Qiagen) for 24 h prior to pre-treatment with nocodazole. Cell lysates were isolated and separated by SDS-PAGE. Western blots were incubated with anti-cyclin B2 (K0189-3, Cell Signaling), anti-ASAH1 (Sigma), and anti-GAPDH (Santa Cruz) antibodies. Protein expression was detected using an ECF western blotting kit (GE Healthcare, Piscataway, NJ) and visualized using a VersaDoc 4000 Imager (Bio-Rad Laboratories, Hercules, CA).

### *A1.2.6. Transient transfection and reporter gene analysis*

Cloning of the human ASAH1 promoter and generation of deletion constructs were previously described (461). MCF-7 cells were sub-cultured into 24-well plates and transfected with 100 ng of pGL3-ASAH1 or pGL3-ASAH1(EREmutant) and 1.5 ng pRL-TK (Promega) using GeneJuice (EMD Biosciences). Some cells were co-transfected with 50 ng pCMV-hER$\alpha$. Twenty-four h after transfection, cells were treated with 20 nM genistein for 16 h and the transcriptional activity of the ASAH1 reporter gene determined using a dual luciferase assay kit (Promega). *Firefly* (pGL3-ASAH1) luciferase activity was normalized to *Renilla* luciferase activity (pRL-TK) and expressed as fold change over the mean of the untreated control group.

### *A1.2.7. Site-directed mutagenesis*

Mutagenesis of the putative ER response element (ERE) at position

-475/-457 of the ASAH1 promoter was carried out using a QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA) and confirmed by sequencing. ERE was disrupted by mutating 4 consecutive residues to alanine (italized) using the following primer set: forward 5'- GGG CAA AGA TGG *AAA A*GG GTG GGA TGT TAC-3', reverse 5'- ACA TCC CAC CCT TTT CCA TCT TTG CCC TCT-3'.

### *A1.2.8. Chromatin immunoprecipitation (ChIP)*

MCF-7 cells were sub-cultured into 150-mm dishes and treated for 1 h with 20 nM genistein and ChIP assays were performed as described previously (158). The purified chromatin solutions were pre-cleared and immunoprecipitated using 5 μg of primary antibody [anti-acetyl-histone H3, anti-RNA polymerase II, anti-phospho-$Ser^{118}$-ERα (clone NL-44), anti-SRC-1, or anti-Sp1] and 30 μL protein A/G Plus agarose (Santa Cruz). Quantitative PCR was carried out using 20% of output, 5% input (diluted 1:4) and the ABsolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific).

### *A1.2.9. Cell proliferation*

For quantitative proliferative assays, MCF-7 cells were seeded into 96-well plates (5 x $10^3$ cells/well) and 24 h later transfected with 75 nM non-specific siRNA oligonucleotides or siRNA against ASAH1 or siRNA against ASAH2 (M-005229-00-0005, Dharmacon/Thermo Scientific) for 24 h. In some experiments, cells were seeded in 96-well plates (5 x $10^3$ cells/well) and 24 h later transfected with an ASAH1 expression plasmid (0.4 μg/well) for 24 h. Cells were then treated with 20 nM genistein, 10 nM G-1, or vehicle (DMSO) for 24 h. After treatment, the cultures were incubated for an additional 6 h in the presence of 5-bromo-2-deoxyuridine (BrdU; 10 μM). Cell proliferation was assayed by BrdU incorporation measurements with an ELISA kit (Roche Applied

Science, Indianapolis, IN). Western blotting was used to confirm reduction of ASAH1 and ASAH2 protein levels 48 h after siRNA transfection. Similarly, ASAH1 overexpression was confirmed by western blotting 48 h after transfection.

### *A1.2.10. Cell viability*

MCF-7 cells were plated into 96-well plates (5 x $10^3$ cells/well) and 24 h later transfected with 75 nM non-specific siRNA oligonucleotides or siRNA against ASAH1 (M-005228-01-0005, Dharmacon/Thermo Scientific). Twenty-four h later, cells were treated for 12, 24, or 48 h with 20 nM genistein or DMSO. Cell viability was assessed using the CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay (Promega) following the manufacturer's instructions. Reduction of ASAH1 protein levels was confirmed by western blotting 48 h after transfection.

### *A1.2.11. Acid ceramidase activity*

MCF-7 cells were sub-cultured into 100-mm dishes and treated for 48 or 72 h with 20 nM genistein and *in vitro* ASAH1 activity assay was performed as previously described (461). TLC plates were visualized by fluorescence scanning on a VersaDoc 4000 imager (Bio-Rad). NBD-dodecanoic acid formation was quantified and normalized to the protein content of each sample.

### *A1.2.12. Statistical analysis*

One-way ANOVA, Tukey-Kramer multiple comparison, and unpaired student t-tests were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Statistically significant differences from a compared value were defined as $p < 0.05$ denoted by asterisks (*) or carats (^).

## A1.3. Results

### *A1.3.1. Genistein induces ASAH1 mRNA expression*

To evaluate the effect of genistein on the transcription of ceramidase genes (ASAH), MCF-7 cells were treated with 20 nM genistein for 24 h. ASAH1 mRNA expression was significantly increased by 2.5-fold in response to low-dose genistein treatment (Figure A1.1A). Interestingly, ASAH2 mRNA expression was also induced by 2.6-fold in response to treatment, while the expression of ACER3 was unchanged (Figure A1.1A). Because aberrant ASAH1 expression has been reported in cancer cells (110,113,716), I focused on the effect of genistein on the transcription of this ceramidase isoform. Experiments to assess the kinetics of the ASAH1 transcriptional response to genistein were carried out by treating cells for 6, 12, and 24 h. As shown in Figure A1.1B, ASAH1 mRNA levels begin to increase after 6 h of genistein stimulation with maximal induction at the 24 h time point.

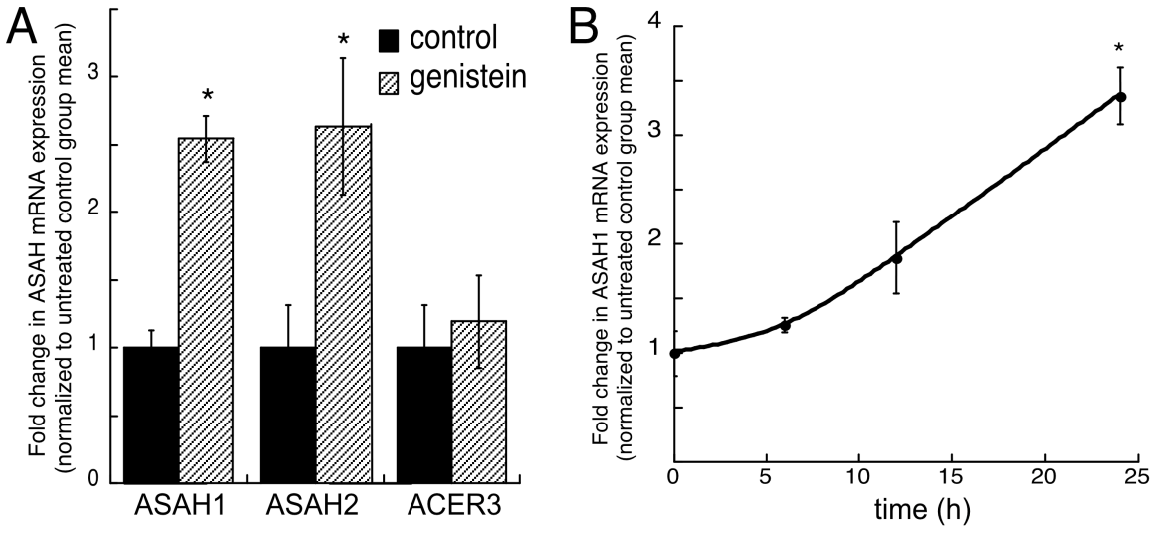



**Figure A1.1.** *Genistein induces ASAH1 transcription*. **A.** MCF-7 cells were treated for 24 h with 20 nM genistein. Total RNA was isolated for analysis of acid (ASAH1), neutral (ASAH2), and alkaline (ACER3) ceramidase mRNA expression by qRT-PCR. **B.** MCF-7 cells were treated for 6, 12, or 24 h with 20 nM genistein and ASAH1 and β-actin mRNA expression were quantified by qRT-PCR. Data graphed are expressed as fold change in ASAH mRNA expression normalized to the mRNA expression of β-actin and represent mean ± SEM of three separate experiments, each done in triplicate. *Statistically different from untreated control group ($p < 0.05$).

### *A1.3.2. GPR30 and ERα mediate genistein-induced ASAH1 transcription*

Genistein activates GPR30-mediated non-genomic signaling in thyroid and breast cancer cells (680-681). Therefore, to investigate the involvement of GPR30 in the up-regulation of ASAH1 expression in response to genistein stimulation, ASAH1 mRNA levels were quantified in MCF-7 cells treated with 10 nM of the high-affinity GPR30 agonist G-1 (720). As shown in Figure A1.2A, G-1 significantly increased ASAH1 transcription by 2.6-fold, which paralleled the fold-increase elicited by genistein. Further, neither genistein nor G-1 was able to induce ASAH1 expression in the presence of the high-affinity GPR30 antagonist G-15 (721) (Figure A1.2A). Significantly, the transcriptional response was not enhanced by G-1 in combination with genistein (Figure A1.2A). Because G-1 and G-15 are not ligands for ER $\alpha$ or $\beta$ (720-721), these data suggest that GPR30 plays a key role in genistein-stimulated ASAH1 transcription. The selectivity of genistein for GPR30 is further supported by the inability of ICI-182780, an ER antagonist (722), to repress genistein-induced ASAH1 mRNA expression (Figure A1.3). ICI-182780 is also a high-affinity GPR30 agonist (684), which is consistent with an increase in ASAH1 mRNA transcript levels observed in ICI-182780 only-treated cells (Figure A1.3).

To determine if GPR30 is required for ASAH1 transcription, GPR30 translation was suppressed (Figure A1.2B, inset) and the effect of reduced GPR30 expression on genistein- and G-1-stimulated ASAH1 mRNA expression was assessed. As shown in Figure A1.2B, neither genistein nor G-1 was able to induce ASAH1 transcription in cells transfected with GPR30 siRNA oligonucleotides. Because genistein can also signal through ERα (698) and GPR30 can work together with ERα in certain $E_2$-mediated

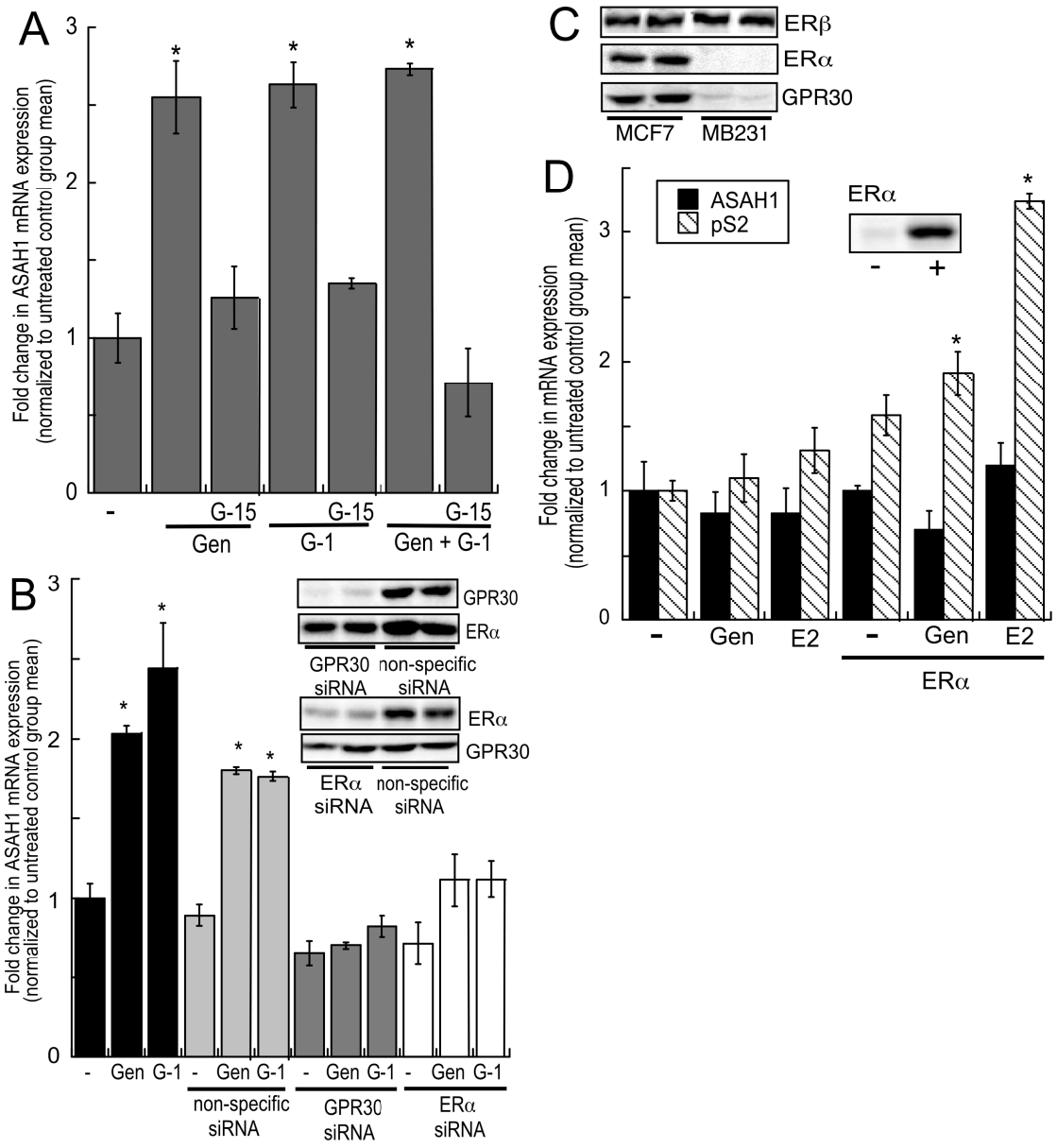



**Figure A1.2.** *GPR30 and ERα mediate genistein-dependent ASAH1 transcription*. **A.** MCF-7 cells were pre-treated for 1 h with 100 or 200 nM G-15 followed by treatment with 10 nM G-1 or 20 nM genistein (gen) for 24 h. Some cells were treated with both G-1 and genistein in the presence or absence of 200 nM G-15. Total RNA was isolated for analysis of ASAH1 mRNA expression by qRT-PCR. **B.** MCF-7 cells were transfected twice with 100 nM GPR30, 75 nM ERα, or 75 nM non-specific siRNA for 48 h (GPR30) or 24 h (ERα) followed by treatment with 20 nM genistein or 10 nM G-1 for 24 h. Total RNA was isolated and ASAH1 mRNA expression was quantified by qRT-PCR and normalized to β-actin. *Inset:* Western blot of GPR30 and ERα protein expression at the time of RNA isolation. **C.** ERα, ERβ, and GPR30 protein quantification in MCF-7 and MDA-MB-231 cells. **D.** ERα was expressed in MDA-MB-231 cells followed by treatment with 20 nM genistein (Gen) or 5 nM $E_2$ for 24 h. ASAH1 and pS2 mRNA levels were quantified by qRT-PCR and normalized to β-actin. *Inset:* Ectopic expression of ERα; (-) control, (+) ERα-transfected cells. Data are graphed as fold change in ASAH1 mRNA expression normalized to the mRNA expression of β-actin and represent mean ± SEM of three separate experiments, each done in triplicate.

activities (707,723-724), I also determined the effect of ERα suppression on ASAH1 transcription. Surprisingly, similar to GPR30, ERα suppression by siRNA (Figure A1.2B, inset) significantly reduced both genistein- and G-1-stimulated ASAH1 transcription (Figure A1.2B). Collectively, these data suggest that both GPR30 and ERα are required for the induction of ASAH1 mRNA expression by genistein.

Consistent with other reports (683,723), MCF-7 cells express ERα, ERβ, and GPR30 protein while MDA-MB-231 cells only express ERβ (Figure A1.2C). Therefore, to further investigate the role of ERα in genistein-mediated ASAH1 transcription, ASAH1 mRNA expression was quantified in genistein-treated MDA-MB-231 cells transfected with an ERα expression plasmid (Figure A1.2D, inset). Significantly, although ectopic expression of ERα was capable of inducing pS2 transcription, a well-known ERα-target gene (also known as TFF1), expression of the receptor was not sufficient to promote ASAH1 mRNA expression in genistein-treated cells (Figure A1.2D).

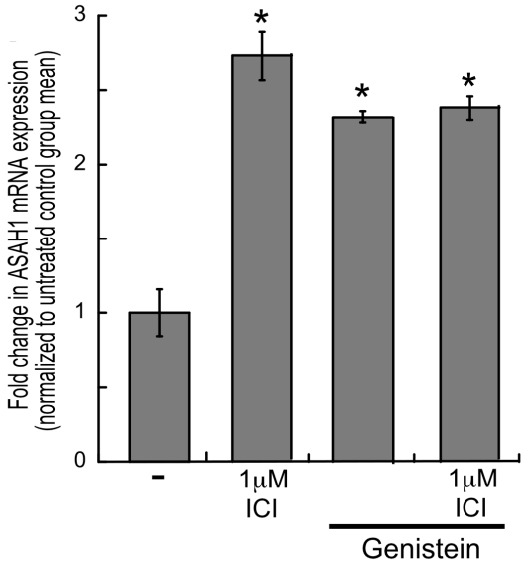



**Figure A1.3.** *ER antagonist effect on genistein-dependent ASAH1 transcription*. **A**. MCF-7 cells were pre-treated for 1 h with 1 μM ICI-182780 followed by treatment with 20 nM genistein for 24 h. Total RNA was isolated for analysis of ASAH1 mRNA expression by qRT-PCR. Data are graphed as fold change in ASAH1 mRNA expression and normalized to the mRNA expression of β-actin. Data graphed represent mean ± SEM of two separate experiments, each done in triplicate. *Statistically different from untreated control group ($p$<0.05).

### *A1.3.3. Genistein-induced ASAH1 transcription occurs via a pertussis toxin-sensitive pathway that requires c-Src and ERK1/2 activation*

GPR30 signaling activates various downstream signaling cascades including ERK and phosphoinositol-3-kinase (PI3K)/Akt pathways (705). Therefore, to determine which kinases are activated by genistein stimulation, the ability of genistein to increase ERK1/2 phosphorylation was tested. As shown in Figure A1.4A, genistein rapidly increased the phosphorylation of ERK1/2 in MCF-7 cells, but genistein did not activate Akt (data not shown). In addition, the effect of genistein on ERK1/2 activation was mimicked by G-1 (Figure A1.4B, top panel) and G-15 prevented ERK1/2 phosphorylation induced by both G-1 (top panel) and genistein (bottom panel) (Figure A1.4B). Additional experiments geared towards identifying effector kinases in the ERK pathway revealed that genistein also activated Raf-1 and c-Src (Figure A1.5A), and that activation of these kinases was required for ERK phosphorylation (Figure A1.5B). Consistent with these western blot data, inhibition of ERK, c-Src, and $G\alpha_i$ activation attenuated genistein-stimulated ASAH1 mRNA expression (Figure A1.4C). Furthermore, in agreement with the modulation of nuclear ERs by membrane-initiated signaling (reviewed in (725)) and ligand-independent activation of ER by ERK signaling (726), both genistein (top panel) and G-1 (bottom panel) rapidly increased the levels of phospho-$Ser^{118}$-ER$\alpha$ by 1.6- and 1.5-fold after 30 min, respectively (Figure A1.4D).

### *A1.3.4. Genistein promotes the recruitment of ER$\alpha$ and Sp1 to the ASAH1 promoter*

To further define the mechanism by which genistein modulates ASAH1 mRNA expression, MCF-7 cells were transfected with a luciferase reporter construct that contains 2.7 kb of the ASAH1 promoter and treated with 20 nM genistein for 16 h. As shown in Figure A1.6A, genistein treatment significantly increased the transcriptional activity of the 2.7 kb reporter gene by 1.8-fold. To identify the genistein-responsive

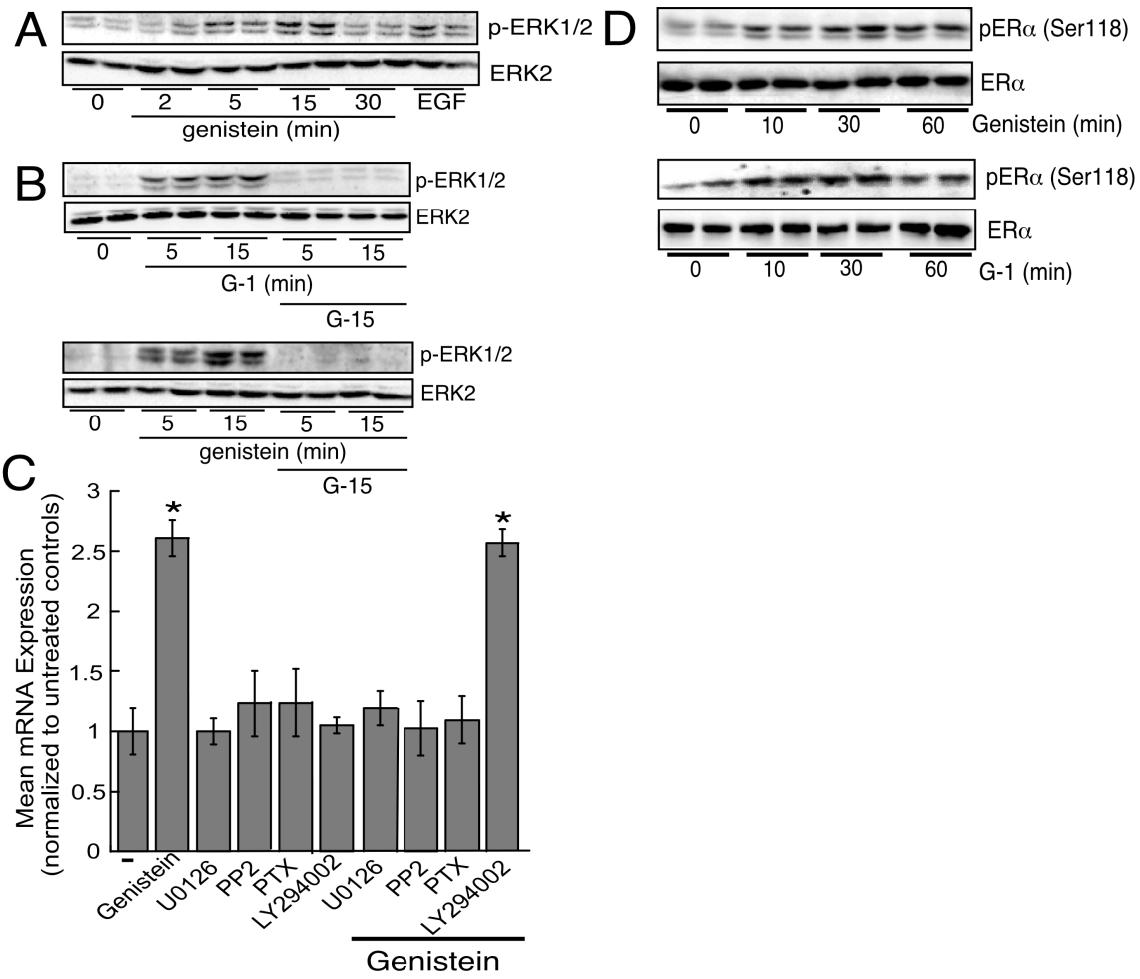



**Figure A1.4.** *Genistein-induced ASAH1 mRNA expression requires G$\alpha_i$, c-Src, and ERK1/2.* **A**. MCF-7 cells were serum-starved for 40 h followed by treatment with 20 nM genistein for 0-30 min or 25 ng/mL EGF for 10 min. Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-phospho-ERK1/2 and ERK2 antibodies. **B.** MCF-7 cells were serum-starved for 40 h, pre-treated with G-15 for 1 h, and then treated with 10 nM G-1 (top panel) or 20 nM genistein (lower panel) for 5 or 15 min. Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-phospho-ERK1/2 and ERK2 antibodies. **C**. MCF-7 cells pre-treated for 1 h with 1 μM U0126, 10 μM PP2, 1 pg/mL PTX, or 10 μM LY294002 followed by treatment with 20 nM genistein for 24 h. Total RNA was isolated for analysis of ASAH1 mRNA expression by qRT-PCR. Data are graphed as fold change in ASAH1 mRNA expression normalized to the mRNA expression of β-actin and represent mean ± SEM of three separate experiments, each done in triplicate. *Statistically different from untreated control group ($p < 0.05$). *D*. MCF-7 cells were serum-starved for 40 h followed by treatment with 20 nM genistein (top panel) or 10 nM G-1 (lower panel) for 0 - 60 min. Lysates were isolated and separated by SDS-PAGE followed by western blotting using antibodies against phospho-Ser118-ERα or ERα.

region(s) on the ASAH1 promoter, different lengths of the ASAH1 promoter (461) were tested for their ability to activate a luciferase reporter construct. While deletion of the region between -2740 bp to -496 bp had no significant effect on the ability of genistein to stimulate reporter gene activity, removal of 250 bp (from the -496 bp construct) completely abolished the genistein response (Figure A1.6A).

Next, I performed *in silico* promoter analysis and identified putative binding sites for Sp1 and ER (ER response element, ERE) within the first 500 bp upstream of the transcription initiation site of the ASAH1 gene (Figure A1.6B). To define key ER transcriptional elements within the genistein-responsive region, a putative ERE at position -475/-457 bp of the ASAH1 promoter (Figure A1.6B) was mutated and genistein-dependent ASAH1 reporter gene activity determined in luciferase assays. As

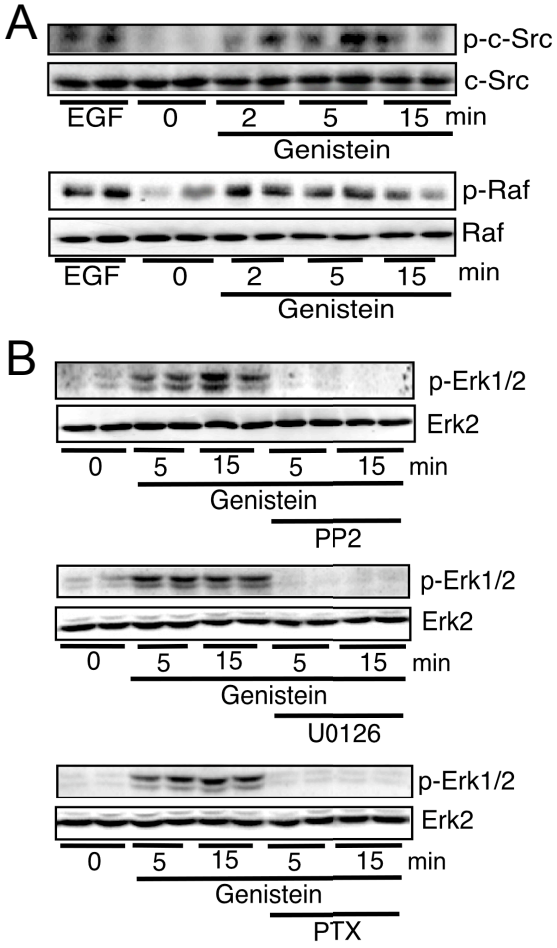
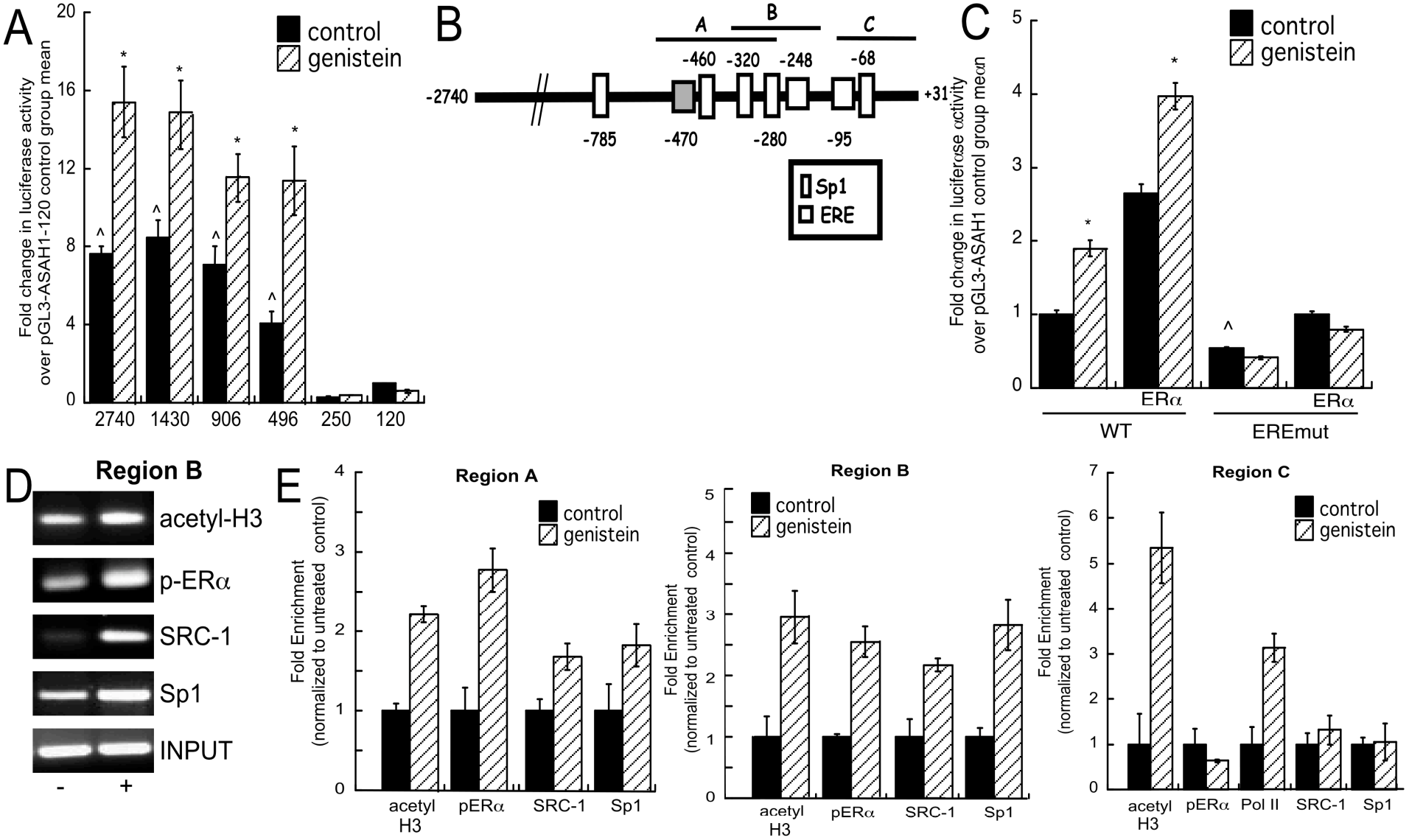



**Figure A1.5.** *Kinase-dependent genistein signaling*. **A.** MCF-7 cells were serum-starved for 40 h and treated with 20 nM genistein for 5 - 15 min or 25 ng/mL EGF for 10 min. Cell lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-phospho-Src and anti-Src (top panel) or anti-phospho-Raf-1 and anti-Raf-1 (bottom panel) antibodies. **B.** MCF-7 cells were serum-starved for 40 h, pre-treated for 1 h with 10 μM PP2 (top panel), 1 μM U0126 (middle panel), or 1 pg/mL PTX (bottom panel), and then treated with 20 nM genistein for 5 - 15 min or 25 ng/mL EGF for 10 min. Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-phospho-ERK1/2 and ERK2 antibodies.

shown in Figure A1.6C, ERα significantly increased the transcriptional activity of the wild type ASAH1 promoter, but failed to increase the reporter gene activity in cells transfected with an ASAH1 reporter plasmid harboring a mutation in the ERE. Interestingly, mutation of this ERE not only abolished the ability of genistein to stimulate promoter activity but also significantly decreased reporter gene activity in untreated cells, suggesting that this ERE may play a role in maintaining basal transcription (Figure A1.6C). I next performed ChIP analysis on chromatin isolated from MCF-7 cells treated for 1 h with 20 nM genistein. Phospho-Ser$^{118}$-ERα was enriched by 2.8-fold at region A (-500/-278) and 2.5-fold at region B (-325/-214) of the ASAH1 promoter in response to genistein (Figure A1.6D and A1.6E). Sp1 and the coactivator SRC-1 were also recruited to regions A and B in response to genistein stimulation (Figure A1.6E). Furthermore, genistein increased acetylation of histone H3 in all regions, indicating that genistein-stimulated phospho-Ser$^{118}$-ERα and Sp1 binding occurs concomitant with histone H3 modification (Figure A1.6E).

### *A1.3.5. Genistein increases ASAH1 protein expression and enzymatic activity*

Next, I determined if genistein also increased protein expression by carrying out western blot analysis of lysates isolated from MCF-7 cells that were treated for 24, 48, or 72 h with 20 nM genistein. As shown in Figures A1.7A and A1.7B, genistein significantly increased ASAH1 protein levels by 1.8- and 2.3-fold after 48 or 72 h treatment, respectively. This increase in ASAH1 protein content resulted in increased ceramidase activity (Figure A1.7C).


A
Fold change in luciferase activity over pGL3-ASAH1-120 control group mean
control
genistein
2740
1430
906
496
250
120
B
A
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C
-460
-320
-248
-68
-2740
+31
-785
-470
-280
-95
Sp1
ERE
C
Fold change in luciferase activity over pGL3-ASAH1 control group mean
control
genistein
ERα
ERα
WT
EREmut
D
Region B
acetyl-H3
p-ERα
SRC-1
Sp1
INPUT
-
+
E
Region A
Region B
Region C
Fold Enrichment (normalized to untreated control)
control
genistein
acetyl H3
pERα
SRC-1
Sp1
Pol II

**Figure A1.6.** *Genistein stimulation promotes the recruitment of ERα and Sp1 to the ASAH1 promoter.* **A.** MCF-7 cells were transfected with reporter gene plasmids (pGL3-ASAH1) containing varying regions of the ASAH1 promoter and a *Renilla* luciferase plasmid (pRL-TK). Twenty-four h after transfection, cells were treated with 20 nM genistein for 16 h and luciferase activity quantified by luminometry. Data are graphed as fold change ± SEM of three separate experiments, each done in triplicate, and normalized to -120 bp construct. Asterisks (*) and carats (^) denote statistically significant differences from untreated control within each transfection group or from untreated -120 bp construct, respectively ($p < 0.05$). **B.** Diagram of putative binding sites for Sp1 and ER within the genistein-responsive region of the ASAH1 promoter. Letters A, B, and C denote regions amplified by each primer set used for ChIP assay. The ERE at position -475/-457 bp that was mutated for further analysis in *'C'* is denoted by a grey-shaded square. **C.** MCF-7 cells were transfected with pGL3-ASAH1-496 (WT) or pGL3-ASAH1-496-EREmutant (EREmut) reporter gene plasmid, pCMV-hERα, and a *Renilla* luciferase plasmid (pRL-TK). Twenty-four h after transfection, cells were treated with 20 nM genistein for 16 h and luciferase activity qu0antified by luminometry. Data are graphed as fold change ± SEM of three separate experiments, each done in triplicate and normalized to untreated WT-transfected controls. Asterisks (*) and carats (^) denote statistically significant differences from untreated control within each transfection group or from untreated WT controls, respectively ($p < 0.05$). *D.* Representative agarose gels of the PCR products from ChIP assays obtained for region B (-325/-214). (-) and (+) denote untreated or genistein-treated, respectively. **E.** MCF-7 cells were treated for 1 h with 20 nM genistein, cross-linked with 1% formaldehyde, and the sheared chromatin immunoprecipitated with antibodies against phospho-$Ser^{118}$-ERα, Sp1, RNA Pol II, acetyl-histone H3, or SRC-1 and recruitment to regions A (-500/-278), B (-325/-214), or C (-123/+34) of the ASAH1 promoter assessed by qPCR. DNA purified was quantified by real time PCR and normalized to the ΔCt values of input DNA. Data are graphed as fold enrichment over untreated control ± STD.

### *A1.3.6. Genistein induces MCF-7 cell proliferation and viability in an ASAH1-dependent manner*

Given that genistein-induced cell growth is mediated by GPR30 signaling (680), I examined the role of ASAH1 in cell proliferation in cells transfected with non-specific, ASAH1, or ASAH2 siRNAs (Figure A1.8A, inset). The increase in BrdU incorporation elicited by both genistein and G-1 was dependent on ASAH1 expression (Figure A1.8A). Although G-1 was able to induce cell proliferation by 1.3-fold in ASAH1-depleted cells, this increase was significantly lower than the 3.4-fold increase observed in untransfected cells (Figure A1.8A). Importantly, suppression of ASAH2 had no effect on genistein- or G-1-induced cell proliferation (Figure A1.8A). Moreover, ASAH1 overexpression potentiated cell proliferation is response to genistein and G-1 stimulation by 1.4- and 2.1-fold, respectively (Figure A1.8B). The role of ASAH1 in genistein-dependent cell proliferation was further supported by MTT assays (Figure A1.8C). Finally, to define the functional significance of ASAH1 on the cell cycle, the effect of ASAH1 silencing on the phosphorylation state of cyclin-dependent kinase 7 (CDK7) and CDK2, and cyclin B2 protein levels in genistein-treated MCF-7 cells was determined. As shown in Figure A1.8D, whereas cyclin B2 expression was increased by 2.3- and 2.5-fold in response to 6 or 12 h genistein treatment, respectively, this increase was attenuated in cells transfected with ASAH1 siRNA oligonucleotides. Although genistein induced the phosphorylation of both CDK7 and CDK2, these events were independent of ASAH1 expression (data not shown).

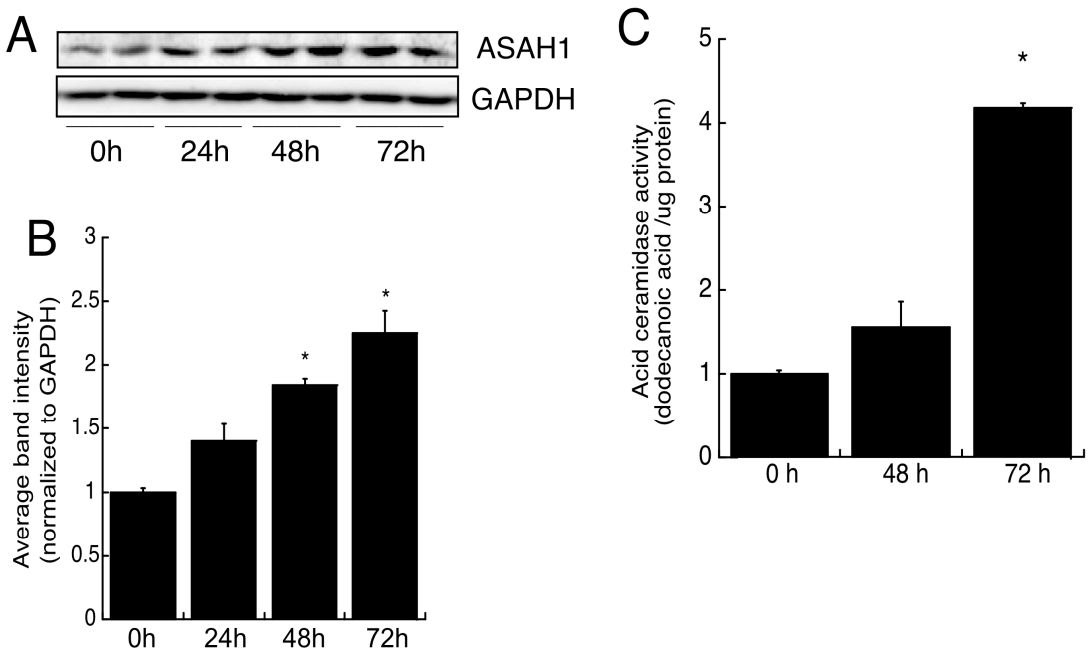


**Figure A1.7.** *Genistein increases ASAH1 protein expression and enzymatic activity*. **A.** MCF-7 cells were treated for 24, 48, or 72 h with 20 nM genistein. Total cell lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-ASAH1 and anti-GAPDH antibodies. **B.** Graphical analysis of data obtained from densitometric analysis of western blots. Data graphed represent mean ± STD of two separate experiments, each done in duplicate. *Statistically different from untreated control group ($p < 0.05$). **C.** MCF-7 cells were treated for 48 or 72 h with 20 nM genistein and cell lysates were isolated for *in vitro* ASAH1 activity assays as described in Section A1.2. TLC plates were visualized by fluorescence scanning. NBD-dodecanoic acid formation was quantified and normalized to the protein content of each sample. Data graphed represents mean ± STD of three separate experiments, each done in duplicate. *Statistically different from untreated control group ($p < 0.05$).

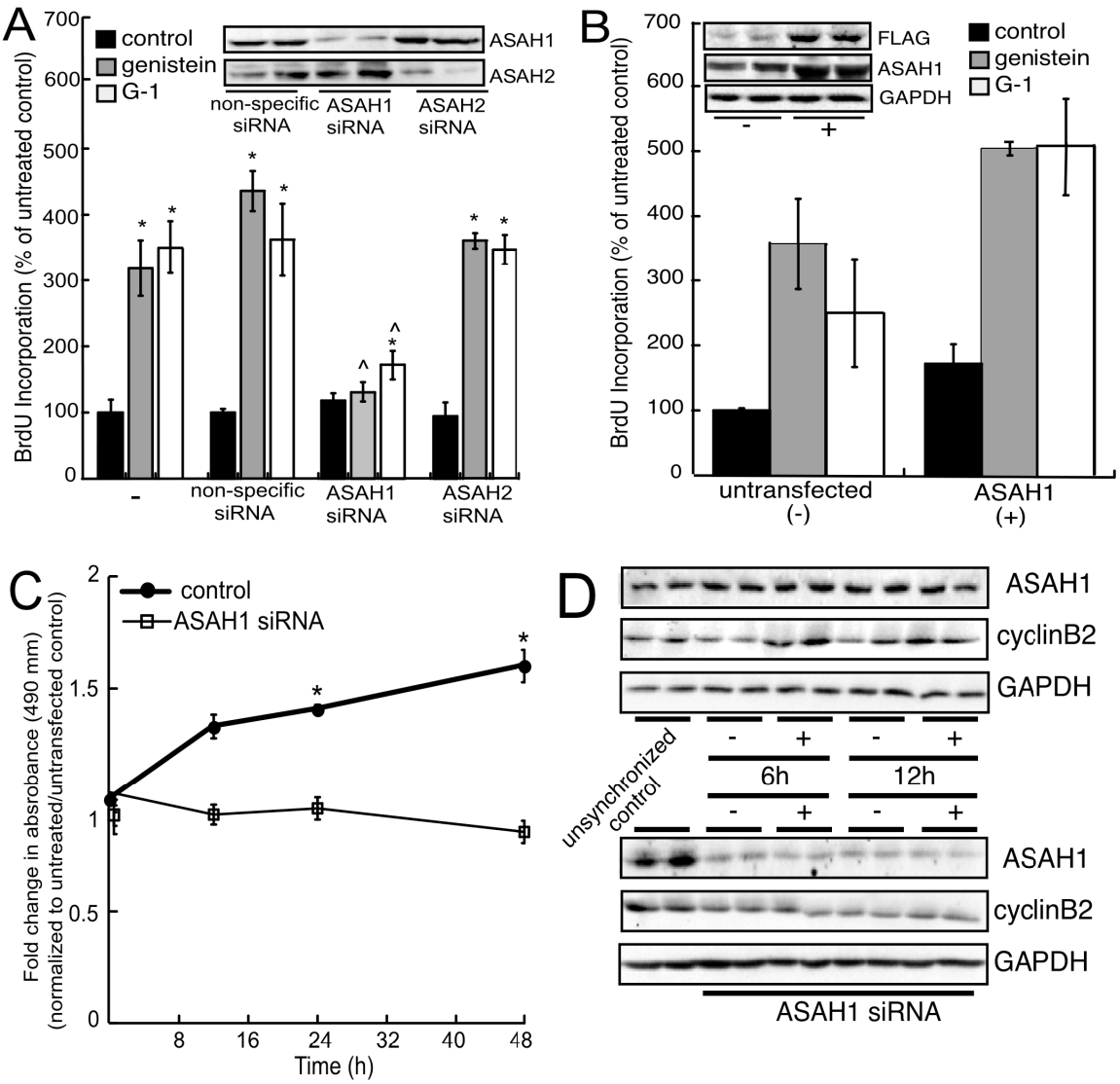


**Figure A1.8.** *Genistein increases cell proliferation, cell viability, and cyclin B2 expression in an ASAH1-dependent manner*. **A**. MCF-7 cells were seeded in 96-well plates at 5 x $10^3$ cells/well and 24 h later transfected with 75 nM ASAH1, ASAH2, or non-specific siRNA for 24 h. Cells were then treated with 20 nM genistein or 10 nM G-1 and 24 h later, cell proliferation was measured with a BrdU incorporation ELISA kit. Data are expressed as mean % of control ± SEM of four separate experiments, each done in quadruplicate. *Inset:* Western blot of ASAH1 and ASAH2 protein expression in siRNA-transfected cells 48 h after transfection. **B.** MCF-7 cells were seeded in 96-well plates at 5 x $10^3$ cells/well and 24 h later transfected with 0.4 μg of an ASAH1 expression plasmid for 24 h. Cells were then treated with 20 nM genistein or 10 nM G-1 and 24 h later, cell proliferation was measured with a BrdU incorporation ELISA kit. Data are expressed as mean % of control ± SEM of three separate experiments, each done in quadruplicate. *Inset:* Western blot of ASAH1 protein expression in control (-) and transfected (+) cells 48 h after transfection. **C.** MCF-7 cells were seeded in 96-well plates and transfected with 75 nM ASAH1 or non-specific siRNA for 24 h. Cells were then treated with 20 nM genistein for 12, 24, or 48 h and cell viability was measured with an MTT assay kit. Data graphed represent mean ± SEM of three separate experiments, each performed in triplicate. Asterisks (*) indicate statistically significant differences from untreated/untransfected or untreated/siRNA-transfected control groups, respectively ($p < 0.05$). **D.** MCF-7 cells were transfected with 75 nM ASAH1 siRNA for 24 h, pre-treated with 200 ng/mL nocodazole for 10 h, and then treated with 20 nM genistein for 6 or 12 h. Total lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-ASAH1, anti-cyclin B2, or anti-GAPDH antibodies. (-) and (+) denotes untreated or genistein-treated groups, respectively. 'Unsynchronized controls' denote cells grown in regular MEM media neither treated with nocodazole nor genistein.

## A1.4. Discussion

Genistein mediates many $E_2$-dependent pathways by binding to ERs (694,700) and it has been reported that this phytoestrogen can promote breast cancer cell growth in a dose-dependent manner (693,698-699,727-728). Although $E_2$-activated non-genomic signaling is well documented (reviewed in (725)), the mechanisms by which phytoestrogens evoke changes in cell growth are less defined. Therefore, I investigated the role of genistein in ASAH1 expression in MCF-7 breast cancer cells.

GPR30 is increasingly recognized as an important mediator of rapid non-genomic estrogenic action (705-706,709,713). I show that 20 nM genistein and the high-affinity GPR30 agonist G-1 induce ASAH1 transcription (Figure A1.1) through a GPR30-dependent (Figure A1.2) ERK1/2 signaling pathway (Figure A1.4). Consistent with my findings, genistein has been shown to activate ERK1/2 through GPR30 in MCF-7 and thyroid cancer cells (680-681). In addition, Maggiolini *et al.* (681) has shown that genistein induces C-FOS gene expression through a GPR30-dependent ERK1/2 cascade that requires c-Src kinase activity.

Importantly, I show that the transcriptional response elicited by genistein requires both GPR30 and ERα (Figure A1.2). Studies have demonstrated that these two receptors act cooperatively to mediate cell proliferation in ER-positive cancer cells (707,724). Although it is possible that GPR30 and ERα are acting in mutually exclusive signaling pathways, three lines of evidence suggest that these two receptors are part of the same signaling cascade: (a) Genistein and G-1 do not have a synergistic effect on ASAH1 transcription (Figure A1.2A), (b) abrogation of either GPR30 or ERα expression by siRNA prevents genistein-dependent ASAH1 gene expression (Figure A1.2B), and (c) ERα suppression inhibits G-1-stimulated ASAH1 gene expression (Figure A1.2B). Reconstitution of this pathway in ERα/GPR30-deficient MDA-MB-231 cells (Figure

A1.2D) revealed that ERα alone is not sufficient to promote ASAH1 transcription in response to genistein, although $E_2$-induced pS2 expression is restored in the presence of the receptor (Figure A1.2D). ER-mediated transcription can be stimulated by ligand-independent mechanisms involving second messenger signaling systems (726). I show that stimulation with genistein and G-1 leads to phosphorylation of ERα at $Ser^{118}$ (Figure A1.4D), a major target of MAPK signaling (726,729). Furthermore, promoter analysis revealed that an ERE at position -475 bp of the ASAH1 promoter is required for ERα-dependent ASAH1 reporter gene activity (Figure A1.6C), suggesting that ERα is recruited to this site following genistein stimulation. Indeed, ChIP studies demonstrated that phospho-$Ser^{118}$-ERα is recruited to the ASAH1 promoter in response to genistein stimulation (Figure A1.6D). Sp1, which has been shown to interact with ERα to transactivate target genes by binding to Sp1xERE motifs (730), is also recruited to the same promoter region (Figure A1.6D). In agreement with these findings, ASAH1 expression was shown to positively correlate with ER status in breast cancer tumors (716) and ER-positive tumors display higher ASAH1 expression (731), supporting a role for ER in the transcriptional regulation of the ASAH1 gene.

Significantly, I show that ASAH1 expression is required for the increase in cell proliferation by genistein (Figure A1.8A) and ASAH1 overexpression potentiates this proliferative response (Figure A1.8B). These data suggest that ASAH1 mediates genistein-stimulated cell proliferation. Moreover, I demonstrate that genistein increases cyclin B2 protein expression in an ASAH1-dependent manner (Figure A1.8D). Cyclin B2 expression during G2/M phase of the cell cycle facilitates the activation of CDK1, regulates mitotic progression (583), and prevents DNA re-replication (732). Thus, the data presented in this Chapter suggests that endogenous ASAH1 mediates progression through the cell cycle in response to low doses of genistein, at least in part, by triggering

cyclin B2 expression. Of note, ACER3 and ASAH2 were both reported to modulate cell proliferation either by upregulating CDK inhibitor $p21^{CIP1/WAF1}$ expression (100) or inducing cell cycle arrest at $G_0/G_1$ and Rb protein dephosphorylation (472), respectively. Genistein has been shown to promote cell proliferation of pancreatic β-cells (733), and breast (681,686,700), thyroid (680), and prostate (734) cancer cells through multiple signaling pathways. Because genistein has a dose-dependent effect on the growth of breast cancer cells (693,698-699), it is noteworthy that the effect of genistein on ASAH1 transcription was only observed at nanomolar concentrations (micromolar concentrations had no effect on ASAH1 transcription, data not shown), the same dosage at which it promotes tumor growth *in vivo* and *in vitro* (686,693-694,703,727-728). Because genistein also induced the transcription of ASAH2 (Figure A1.1A) and SPHK1 (data not shown), it is likely that this phytoestrogen may promote sphingolipid metabolic changes by regulating the expression of multiple sphingolipid genes. Indeed, mass spectrometric analysis revealed that genistein evoked a 1.3-fold increase in the cellular concentration of S1P (Lucki and Sewer, unpublished observations). Total Cer levels were not significantly altered by genistein stimulation while SM levels modestly increased by 1.2-fold in genistein-treated cells (Lucki and Sewer, unpublished observations). Given the complexity of the sphingolipid metabolic pathway and its many metabolites, temporal genomic transcriptional and lipidomic analysis is required to pinpoint the mechanisms by which genistein alters sphingolipid concentrations. Gupta *et al.* (246) recently reported a quantitative model of the sphingolipid pathway that illustrates the complexity of sphingolipid flux alterations in response to pharmacological perturbations. Nonetheless, because S1P activates cell growth-related pathways in a paracrine/autocrine manner by binding to S1PRs (621,735) (Figure 1.8), it is possible that genistein also promotes S1P secretion. Given that SPHK1 is overexpressed in breast cancer cells (736), it is likely that an increase in ASAH1 activity could lead to increased S1P production. Of note, $E_2$

was shown to promote breast cancer cell proliferation by activating SPHK1 (737) and Takabe *et al*. (192) recently demonstrated that $E_2$ induces ERK1/2 activation in MCF-7 cells by stimulating SPHK1 activity and promoting a rapid secretion of S1P in an ER$\alpha$-dependent manner. Further studies are required to establish that genistein evokes the export of S1P via a similar mechanism. Nonetheless, because $E_2$ treatment mirrored the stimulatory effects of genistein on ASAH1 transcription and protein expression (data not shown), the regulation of ASAH1 expression by this phytoestrogen adds to the current understanding of the molecular mechanisms underlying $E_2$-dependent mammary cell proliferation. Because Cer degradation is the only source of cellular SPH (42,104), ASAH1 directly regulates SPHK1 substrate availability. Thus, the effect of $E_2$ on SPHK1 activity would benefit from increased ASAH1 expression. Further, long-term exposure to genistein was recently shown to enhance $E_2$ responsiveness in rat uterus (738) and reverse the inhibitory effects of tamoxifen (739-740). Therefore, I hypothesize that low doses of genistein may have a detrimental additive effect to $E_2$ *in vivo* by promoting sphingolipid-mediated breast cell growth.

In conclusion, I propose a model whereby genistein induces ASAH1 gene expression by activating a GPR30-dependent pathway that culminates in ERK1/2 phosphorylation (Figure A1.9). ERK1/2 activation induces the phosphorylation of ER$\alpha$ and the binding of a complex containing the receptor, Sp1, and SRC-1 to the ASAH1 promoter. ASAH1 transcription is followed by increased protein expression and enzymatic activity, which results in increased S1P production. S1P can then be exported (621) and activate proliferative pathways by binding to cell surface S1PRs (735) (Figure A1.9). Concomitantly, ASAH1 mediates cyclin B2 expression, which drives mitotic progression and, consequently, cell growth. Further studies are needed to determine if S1P is involved in genistein-dependent cyclin B2 expression. In light of the growing interest in the role of phytoestrogens in cancer progression and treatment, and the

development of chemotherapeutic strategies to modulate sphingolipid metabolism, my results provide evidence for the integral role of ASAH1 in maintaining cellular proliferative capacity of ER-positive breast cancer cells.

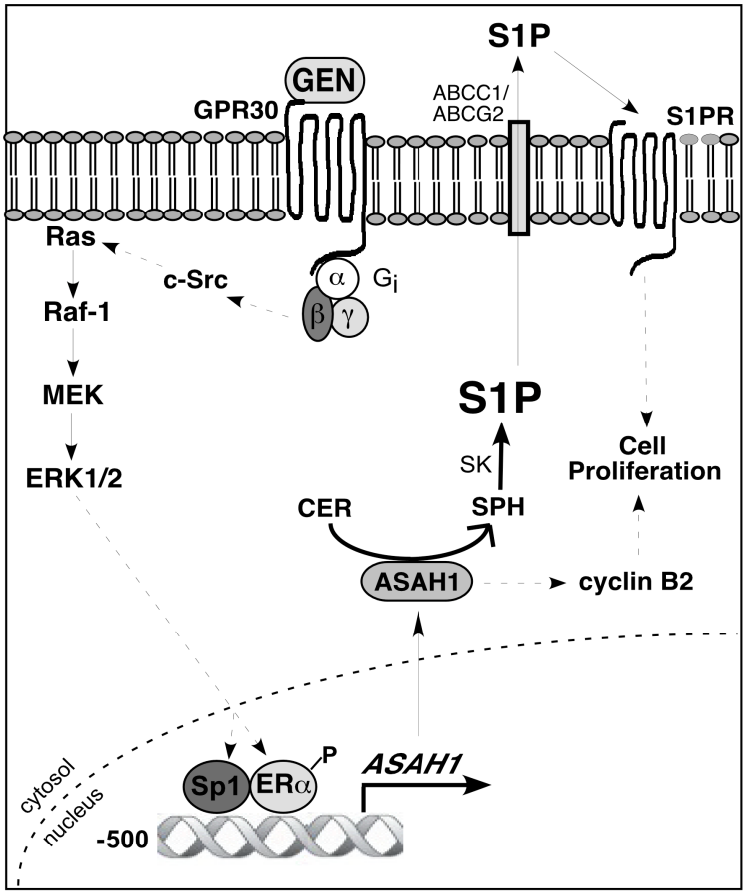


**Figure A1.9.** *Model pathway for genistein-induced ASAH1 transcription in MCF-7 cells.* Genistein (GEN) activates GPR30, which signals through $G\alpha_i$ to activate c-Src and subsequently the MAPK pathway. ERK1/2 activation promotes the phosphorylation of ERα and its recruitment to the ASAH1 promoter. Induction of ASAH1 gene transcription leads to an increase in protein expression and subsequent upregulation of enzymatic activity, which results in the degradation of ceramide (CER) into sphingosine (SPH) and its phosphorylation into sphingosine-1-phosphate (S1P). S1P is exported from cells via the ABC transporters ABCC1 and ABCG2 and acts in a paracrine/autocrine matter to activate proliferative signaling cascades by binding to cell-surface S1P receptors (S1PR). Concomitantly, ASAH1 mediates cyclin B2 expression.

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